Behavioural and molecular genetic studies of Oreochromis species and their hybrids.

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Behavioural and Molecular Genetic Studies of *Oreochromis* Species and their Hybrids

by

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SUMMARY

The tilapiine cichlids of the genus *Oreochromis* are an increasingly important component of aquaculture in many countries. Aquaculture in Malawi is expanding rapidly and its importance may increase if local cichlid fisheries continue to decline. There is concern that sympatric species (*O. shiranus* and *O. karongae*) are hybridizing in farm ponds. The ease with which *Oreochromis* species hybridize is of much concern to the maintenance of their biological diversity.

Mate choice and courtship behaviour were investigated in the allopatric species, *O. mossambicus* and *O. spilurus,* and their hybrid. Pure-bred females largely mated assortatively and behaved differently to conspecific and heterospecific males. Females mated with heterospecifics if conspecific males were not available. Hybrid females showed a preference for *O. mossambicus*. Differences in behaviour largely predicted mate choice in pure-bred fish.

O. karongae is one of three species belonging to the unique *Oreochromis (Nyasalapia)* species flock of Lake Malawi ('chambo'). The incidence of hybridization in farm ponds was examined using allozyme, RAPD and morphological analysis. Markers that distinguished between *O. shiranus* and chambo were found with both molecular genetic techniques. Markers for each chambo species were not detected. *O. shiranus* and chambo were discriminated using eight morphometric variables. Chambo species were discriminated using morphometrics, meristics and colouration.

Hybrids of *O. shiranus* and chambo were detected in all ponds sampled from three sites in Malawi. No F_1 hybrids were detected and introgression was common. Discrepancies in fish identification illustrates the merits of using several techniques when identifying post-F_1 hybrids. Morphological analysis was least effective in identifying hybrids. The implications of hybridization and possible escapes into the wild are discussed in relation to the impacts on aquaculture and on the unique cichlid species endemic to Lake Malawi. Recommendations for the management of *Oreochromis* in aquaculture are considered in relation to ecological and social issues. Processes that maintain reproductive isolation between *Oreochromis* species are discussed.

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CHAPTER 1
GENERAL INTRODUCTION

1.1 INTRODUCTION

Tilapiine cichlids, largely of the genus *Oreochromis* (the maternal mouthbrooders), constitute an increasingly important source of protein throughout the world. Many introductions have been made within and outside their native range, frequently leading to hybridization between previously allopatric and sympatric forms. The ease with which *Oreochromis* species hybridize, sometimes producing offspring that are as fertile and as viable as the parental species, complicates the recognition of species according to the biological species concept in which reproductive isolation is a fundamental component. In many cichlid species post-mating barriers are not well developed and breeding behaviour (ethological isolation) plays a predominant role in maintaining reproductive isolation between sympatric species (Baylis 1976a; Crapon de Caprona 1986). Behavioural studies, on pure-bred species and their hybrids, have contributed greatly in the identification of isolating mechanisms (in many taxa), and thereby, to an understanding of the biological processes of speciation (Butlin & Ritchie 1994; Dall 1997). In cichlids, interspecific mate recognition is augmented by an advanced communication system, in which sexually selected characters such as courtship colouration and behaviour are particularly important (Baerends & Baerends-van Roon 1950; Baylis 1976b; McKaye *et al.* 1993). Nevertheless, ethological and other isolating mechanisms operative among sympatric species, commonly do not exist amongst formally allopatric species.

Hybridization is particularly prevalent in fishes, as exemplified by Schwartz's studies (1972, 1981) in which he compiled a list of 3759 references dealing with natural and artificial hybridization in fishes (cited in Avise 1994). Weak ethological isolating mechanisms and susceptibility to secondary contact between recently evolved forms, are two of several characteristics of fishes which account for this common phenomenon. Extensive hybridization, where hybrids are fertile, may lead to the transfer and incorporation of novel genetic variants into the genepool of a population (i.e. introgression). Introgressive hybridization is a phenomenon not only confined to the interbreeding of distinct species (interspecific), but also occurs within species (intraspecific). In fact, the term 'hybridization' is employed in a broad sense to include crosses between genetically differentiated forms regardless of their current taxonomic status (Avise 1994). Hybridization is, therefore, the interbreeding of unlike individuals or populations either under

human control or in nature, and can range from crossing between virtually identical individuals to crossing between individuals of different species (Mayr 1963; Schonewald-Cox *et al.* 1983). Understanding and illustrating the evolutionary consequences of hybridization has been greatly facilitated through the implementation of molecular genetic techniques (e.g. Dowling & DeMarais 1993). Prior to the development of molecular genetic techniques, the use of morphological characters was essentially the only method available for identifying hybrids. Many cases of hybridization have been verified and characterized further using molecular markers, and molecular genetic analysis has lead to many new and valuable insights into animal behaviour.

The aim of this opening chapter is to provide a background to the rationale of the present study by highlighting four relevant issues:

1) *Species concepts and reproductive isolation* - Reproductive isolation, fundamental to the process of speciation, allows gene pools to evolve independently and is therefore a principal component of many species concepts. Taxa in which a capacity to hybridize is frequently realised, present a problem in the application of species concepts where reproductive isolation is a fundamental component. Several mechanisms exist by which species may remain reproductively isolated. Of particular concern to this study is the role of breeding behaviour in the evolution and maintenance of reproductive isolation.

2) *Hybridization and evolutionary change* - Processes which lead to the breakdown of reproductive isolation are introduced, with reference to the characteristics of fish species which renders them particularly vulnerable to hybridization. The role of hybridization in evolutionary change is examined and salient topics of hybridization are introduced, including, introgression, hybrid zones and speciation. The application of behavioural experiments and molecular genetic techniques in the examination of reproductive isolation and hybridization processes is introduced.

3) *Methods of detecting hybridization* - An examination of the techniques available for detecting hybridization (morphological and genetic based), in which the advantages and disadvantages of particular approaches will be highlighted.

4) *The biology and aquaculture of tilapia* - The biology and aquaculture of tilapia are introduced. Of particular interest is the breeding behaviour of tilapia and their propensity to hybridize in relation to the problems associated with their aquaculture. The aquaculture of *Oreochromis* species in Malawi is expanding rapidly and may become increasingly important if the local fishery continues to decline. Hybridization between *Oreochromis* species in farm ponds is of great concern because of the unique tilapiine species flock, the *Oreochromis (Nyasalapia)*, of Lake Malawi.

1.2 SPECIES CONCEPTS AND REPRODUCTIVE ISOLATION

1.2.1 The Species concept

Several species concepts have been proposed, most of which involve the perception of conspecific populations within which genetic exchange potentially takes place. Dobzhansky (1937) was the first to define species in terms of interbreeding and reproductive isolation; other definitions are variants of the same theme known as the biological species concept or the isolation species concept (ISC). Under this concept, Mayr (1970) characterized species as 'groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups'. Reproductive isolation, of concern in a study of hybridization, is used as the fundamental criterion to define species because it allows gene pools to evolve independently of each other (Gosling 1994). Species of *Oreochromis*, the subject of this study, interbreed (or have the potential to interbreed), but usually due to anthropogenic intervention in which allopatric species are brought together through introductions, transfers and aquaculture. Naturally sympatric species may also hybridize in captivity. Indeed, as stated by Mayr (1963), the "occurrence of some hybridization does not, however, necessarily mean that the respective populations are not good species. Many good species of animals are capable of producing hybrids in captivity, but never interbreed in nature". Nevertheless, strict application of the ISC would unite taxa which hybridize into a single biological species. Similarly, allopatric taxa which hybridize in a zone of contact (i.e. hybrid zone), would be considered as a single species despite the maintenance of morphological, ecological and genetic integrity on either side of the hybrid zone (Section 1.3.5) (Barton & Hewitt 1985; Gosling 1994). Furthermore, taxa which have arisen through hybridization (Section 1.3.6) also do not fit the isolation concept (Bullini 1994).

Further problems arise in the application of the ISC because it implies there is a close correspondence between breeding groups and morphologically and ecologically discrete units. For example, different species can be morphologically identical (sibling species) and reproductively isolated. Alternatively, as in the case of the haplochromine cichlids of Lake Victoria (Meyer *et al.* 1990), some species may show morphological diversity but are genetically very similar. The property of sharing genes within a species and the property of isolation from other species are difficult criteria to apply in practice (Butlin & Ritchie 1994). There is no clear distinction of how much genetic exchange disqualifies populations from status as separate biological species. There are no clear solutions to these problems because divergence and speciation normally are gradual processes, and because levels of genetic exchange can vary along a continuum (Avise 1994). Other

complications in applying the ISC arise from the need to distinguish the evolutionary origins of reproductive isolating barriers from their genetic consequences. Furthermore, since the ISC is applicable only to sexually reproducing organisms, a large portion of taxa is excluded (Gosling 1994; Genermont 1995).

Several alternatives to the ISC have been proposed. The recognition species concept (RSC) of Paterson (1980, 1985) also views conspecific populations as a field for gene recombination, although species are defined in terms of recognition of conspecifics rather than by isolation from other species. Under the RSC, species are defined as 'the most exclusive group of individual biparental organisms which share a common fertilization system'. Paterson (1980, 1985) referred to this system as the 'specific-mate-recognition system' (SMRS). Unlike the ISC, the function of isolating mechanisms (SMRS) are viewed more as facilitating reproduction among members of a species rather than acting as barriers to gene flow. Assortative mating in allopatric species would provide evidence of different SMRS (explored in Chapter 2). Nevertheless, as with the ISC, strict application of the RSC would unite taxa which hybridize. Indeed, because of its emphasis on reproduction, the RSC suffers from the same limitations as the ISC. The RSC and ISC differ when post-zygotic barriers to gene flow (i.e. barriers which act after fertilization) are considered. According to the ISC two populations are considered separate species if their hybrids were completely inviable or infertile. If these two populations had the same SMRS, such that hybrids are produced in nature despite having zero fitness, the RSC would consider them conspecific (Paterson 1985; Butlin 1987b). Butlin & Ritchie (1994) suggested that the RSC may go too far in denying that heterospecific interactions may exert any significant selection on mating signals or that processes occurring after fertilization may also be important.

A model which does not place sole emphasis on reproduction is the cohesion species concept (CSC) of Templeton (1989), which concentrates on processes which cause groups of organisms to be similar to one another. Under the CSC, species are defined as 'the most inclusive population of individuals having a having a potential for cohesion through intrinsic cohesion mechanisms'. The major classes of cohesion mechanisms are genetic exchangeability (factors that define the limits of spread of new genetic variants through gene flow) and demographic exchangeability (factors that define the fundamental niche and the limits of spread of new genetic variants through genetic drift and natural selection) (Avise 1994). The CSC deals with a broad array of evolutionary forces, thereby facilitating the study of the speciation process. However, despite this and other advantages (see Gosling 1994; Avise 1994; Genermont 1995), the CSC

suffers from the same operational difficulties as the ISC and RSC (Gosling 1994).

The phylogenetic species concept (PSC) of Cracraft (1983) emphasizes the most general aspect of taxonomic diversification, namely differentiation (morphological, ecological and genetic), some of which results in reproductive isolation and some of which does not. According to the PSC, a species is 'an irreducible (basal) cluster of organisms, diagnosably distinct from other such clusters, and within which there is a parental pattern of ancestry and descent'. This concept, in which individuals share a common evolutionary fate through time (i.e. are monophyletic) can be applied to both sexual and asexual organisms, to allopatric and sympatric populations and to living and extinct groups (Gosling 1994). This definition is very similar to the 'evolutionary' species definition of Simpson (1951), which was favoured by Bullini (1994) in the application of a species concept to hybrid species. The PSC has been criticised because it does not deal with the evolutionary mechanisms responsible for cohesion and, therefore, does not provide an adequate framework for integrating population genetic factors into the species concept (Gosling 1994). A further difficulty with the PSC is deciding which traits are the important ones in defining species (Gosling 1994), since a different trait may apply to different groups. In *Oreochromis,* classification is largely based on morphological characteristics (such as meristic, breeding colouration and pharyngeal structure) (Trewavas 1983). The PSC is perhaps a more useful working definition of *Oreochromis* species, since emphasis is on differentiation and not on reproductive isolation. However, such characters may be especially vulnerable to convergence.

1.2.2 Reproductive isolating mechanisms

Dobzhansky (1951) first recognised the importance of isolating mechanisms, that is, "the biological properties of individuals which prevent the interbreeding of populations that are actually or potentially sympatric" (Mayr 1970). The term 'isolating mechanisms' suggests that the characters evolved for the function of preventing gene flow or that they are adaptations for maintaining the species integrity (i.e. reinforcement), but it is now generally accepted that isolation is an effect, not a function, of characters (Butlin 1987a, b; Paterson 1978). Reproductive isolating mechanisms (RIMs) are, therefore, generally viewed as a non-adaptive by-product of genetic divergence that develop between geographically separated populations (Butlin 1987b, 1989; Paterson 1980, 1985). Divergence results from processes such as genetic drift, adaptation to environmental differences and sexual selection (Templeton 1981; West-Eberhard 1983; Butlin 1989). It is much easier to show that differences among species cause reproductive isolation than to understand the

evolutionary forces that produces these differences (Coyne & Charlesworth 1997). Many species differences contribute little or nothing to reproductive isolation, and hybridization experiments have been instrumental in distinguishing between the genetics of speciation and the genetics of species differences (Templeton 1981). Examples of all standard types of isolating barriers have been revealed by hybridization experiments. Furthermore, hybridization experiments often reveal information about the regulatory differences between species at the developmental or molecular level. For example, hybrids between the sibling species *Drosophila melanogaster* and *D. simulans,* display 'nucleolar dominance' in which one set of ribosomal genes are not transcribed (Rieger *et al.* 1979, cited in Templeton 1981).

Reproductive isolation is more likely to evolve in a series of small steps than in a single genetic revolution (Barton & Charlesworth 1984). Often several isolating factors between species exist simultaneously, and total isolation between two species is normally due to a multitude of isolating mechanism which interact. Indeed, evidence from experimental crosses and hybrid zones shows that reproductive isolation usually involves many genes (Barton & Charlesworth 1984; Danzmann *et al.* 1993b; Coyne & Charlesworth 1997), although many converse cases exist. Speciation is not always due to a large number of genes, in some cases the genetic basis of isolation can involve only one or a few major segregating units ('Type II' ; Templeton 1981). Complete reproductive isolation is the end point of the speciation process, although many diverging taxa are in a period where they are still capable of interbreeding (Grant & Grant 1996).

Reproductive isolating mechanisms are classified as prezygotic or postzygotic barriers (Mayr 1970; Avise 1994). Prezygotic barriers impede or prevent hybridization of members of different populations, and thereby prevent wastage of gametes, whereas, postzygotic barriers reduce the viability of hybrids that have arisen. Alternatively, RIMs can be classified as intrinsic or extrinsic barriers. Intrinsic barriers are due to biological features, which are often genetically determined, and extrinsic barriers are those imposed by features of the environment, primarily geographic distance (Butlin & Ritchie 1994). Changes in species distribution may overcome extrinsic barriers; an issue central to this study since post-zygotic barriers are not well developed in *Oreochromis* (and many cichlid) species. Reproductive isolation in sympatric species of cichlids, which often produce fertile hybrids, is largely dependent on prezygotic barriers.

1.2.2.1 Prezygotic barriers

Paterson (1980, 1985) suggested the term 'specific-mate-recognition system' (see Section

1.2.1), rather than RIMs, to emphasize the primary role of characters in prezygotic isolation in promoting efficient mating and fertilization within a species. In cichlid species, particularly the mouthbrooding species in which there are no pair-bonds and courtship can be very brief, sexually selected characters such as courtship colouration and behaviour play an important role in mate recognition (Baerends & Baerends-van Roon 1950; Baylis 1976a, b; McKaye *et al.* 1993). Ethological isolation, in reference to all species in which behaviour maintains reproductive isolation, acts through specific courtship or displays that only conspecifics are receptive to, via visual, acoustic, tactile and/or chemical stimuli. The signals that function as isolating mechanisms may also serve various other functions, which may further reinforce the isolating mechanism, such as advertising the presence of potential mates and synchronizing mating activities (West-Eberhard 1983). Courtship involves an exchange of stimuli between male and female continuing until both have reached a state of physiological readiness in which successful copulation can occur. The basic pattern of courtship is often the same in closely related species and the differences are often quantitative rather than qualitative (Mayr 1970).

Behaviour is probably more liable, and subject to modification, than any other means of reproductive isolation in vertebrates, and can thus evolve more quickly than other isolating mechanisms (Baylis 1976a). In many fish species (including cichlids), which fail to show postzygotic isolation, the primary RIMs are ethological (e.g. catastomid fishes, *Catastomus* (Nelson 1968) and, marine fish of the genus *Bathygobius* (Rubinoff & Rubinoff 1971)). Nevertheless, ethological isolating mechanisms operative among sympatric species, commonly do not exist between con-generic allopatric species. For example, in Lake Apoyo, the sympatric cichlid species *Cichlasoma citrinellum* and *C. zaliosum* are ethologically isolated, but behavioural isolation is incomplete between the allopatric species *C. citrinellum* and *C. labiatum* (Baylis 1976a). Similarly, a lack of complete behavioural barrier has been demonstrated between allopatric species of Panamanian *Bathygobius* (Rubinoff & Rubinoff 1971). Furthermore, ethological barriers commonly do not prevent heterospecific matings when conspecifics are rare or absent.

Some prezygotic RIMs involve interactions between species and their environment. For example, populations may remain isolated due to habitat restrictions or preferences, and within these habitats most matings take place (Bush 1994). Such ecological or habitat isolation often reinforce other isolating mechanisms. However, habitat isolation is not a very effective isolating mechanism in mobile animals and, as will be further discussed, hybridization is relatively frequent when human or other disturbances breakdown borders between habitats. Temporal isolation, where

matings take place at different times (e.g. seasonally or diurnally), have probably arisen as a by-product of selection for optimal niche utilization and often only function secondarily as RIMs, such as in closely related species where breeding seasons often overlap (Mayr 1970).

The two final prezygotic RIMs to be introduced, in which mating occurs but fertilization is unsuccessful, can be viewed as postmating RIMs since the wastage of male gametes is not avoided. Gametic incompatibility or mortality, or mechanical difficulties, are RIMs not commonly found in fishes. Gametic incompatibilities, in which gamete transfer is successful but fertilization does not occur, are due to processes such as an antigenic reaction to sperm. Mechanical isolation, in which the transfer of male gametes is prevented, usually involves the incompatibility of copulatory apparatus, and is commonly seen in insect taxa (e.g. Albuquerque *et al.* 1996).

1.2.2.2 Postzygotic barriers

If fertilization is successful, reproductive isolation may be maintained through the reduced viability or fertility of the F_1 hybrid, rendering it ecologically and ethologically inferior as compared to the parental species. Postzygotic RIMs include F_1 hybrid inviability or sterility, biased sex ratios and unisexual F_1 hybrids, as illustrated by sunfish (*Lepomis*) (Avise & Smith 1974; Bruson & Robinette 1987). Some interspecific crosses of tilapia produce all or nearly all-male progeny due to different systems of sex determination (e.g. *Oreochromis niloticus* x *O. aureus*); a phenomenon which has been taken advantage of by aquaculturists to prevent uncontrolled reproduction (Section 1.5.4.2). Hybrids may be genetically unstable, either through genetic incompatibilities or structural incompatibility of the parental genome, and may lead to severe or lethal physiological disturbances. However, reciprocal heterospecific crosses do not always produce progeny of the same fitness. For example, female *Haplochromis nubilus* x male *H. burtoni* produce lethal hybrids which do not survive the larval stage, whereas the reciprocal cross, female *H. burtoni* x male *H. nubilus*, produce fertile hybrids (Crapon de Caprona & Fritzsch 1984). F_1 hybrid sterility completely prevents backcrossing and introgression. If the F_1 hybrid is fertile, the F_2, backcross, or later-generation hybrids may have decreased viability or fertility due to various imbalances of their gene complexes (Avise 1994). The occurrence of postzygotic reproductive isolation generally increases with increasing genetic divergence, both in chromosome structure and at individual loci. Chromosomal divergence decreases the probability of normal mitosis in hybrid zygotes, whereas increase divergence at individual regulatory and structural loci probably results in disruptions to normal development and physiological fitness (Verspoor & Hammer 1991).

1.3 HYBRIDIZATION AND EVOLUTIONARY CHANGE

1.3.1 The breakdown of isolating mechanisms

Organisms, of many taxonomic groups, retain the anatomical and physiological ability for hybrid production, despite being separated for long periods of evolutionary time. The slower pace of chromosomal evolution and/or a hypothesized lower rate of change in regulatory genes are perhaps the reason why some taxonomic groups, such as birds and frogs, have lost the potential for interspecific hybridization relatively slowly (Pragner & Wilson 1975). However, although some organisms retain a potential for hybridization, another issue is how often reproductive isolating mechanisms fail and hybridization occurs. The frequency and extent of hybridization varies along a continuum (Avise 1994). At one extreme, hybridization may be rare and confined primarily to the production of F_1 hybrids, where distinct populations persist and genetic integrity remains intact. In this scenario, hybridization may have little or no evolutionary consequence. A rare occurrence of hybridization may be due to strong assortative mating, or low hybrid viability or fertility. For example, selection against young-of-year hybrids between the shiners *Luxilus cornutus* and *L. chrysocephalus*, ensures that the ratio of forms may remain stable (Gleason & Berra 1993). At the other extreme, hybridization and back-crossing may be extensive, due to poor reproductive isolation and high hybrid viability and fertility. Hybridizing taxa may merge completely into a single panmictic genepool or 'hybrid swarm', as observed in subspecies of the bluegill sunfish (*Lepomis macrochirus*) (Avise & Smith 1974). The evolutionary consequences of introgressive hybridization are potentially great. It may result in the loss of distinct species or population, and essentially 'reverse' the speciation process, but may also result in the formation of a new species (Section 1.3.6). However, the role of hybridization in the evolutionary diversification of animals largely remains unclear (Dowling & Secor 1997).

Natural hybridization is not a random phenomenon, there are certain factors that facilitate the breakdown of isolating mechanisms (Mayr 1970). Artificial crossing shows that fish have a high inherent capacity to hybridize, even when the taxa crossed are divergent at the species level or higher. The capacity to hybridize has frequently been shown in aquaria (Hubbs 1955), but is also evident in nature (Schwartz 1972, 1981; Verspoor & Hammer 1991). Several characteristics of fishes may account for the common occurrence of natural hybridization: external fertilization (simultaneous spawning and 'sneaky' mating), weak ethological isolating mechanisms, unequal abundance of the two parental species, competition for limited spawning habitat and susceptibility to secondary contact between recently evolved forms. The weakness of postzygotic barriers in

Oreochromis species, in which hybrids are often as fertile as the parental species, combined with the frequency in which these species are introduced and translocated for aquaculture and to supplement fisheries (Welcomme 1966; Daget & Moreau 1981; Ogutu-Ohwayo & Hecky 1991), makes them especially vulnerable to hybridization. Furthermore, *Oreochromis* readily interbreed when conspecifics are rare or absent; a situation which may arise in aquacultural ponds and when they are introduced into the range of an established species.

The inherent capacity of fish to hybridize is affected to varying degrees by local habitat (Campton 1987). For example, the high hybridization rate between Atlantic salmon (*Salmo salar*) and brown trout (*S. trutta*) in the narrow Swedish River Grönån, may be explained by the limited spatial segregation between the two species, due to a lack of separate spawning grounds, in combination with high population densities and overlapping spawning times (Jansson *et al.* 1991). Fertilization of eggs by sneaking of mature male parr has also been suggested as a factor promoting hybridization (Crozier 1984). Rarity of one parental species, particularly at the margin of the normal geographic range of one of the two parental species, may lead to the production of hybrids due to a shortage of conspecific mates (Hubbs 1955). Hybridization of sunfish (*Lepomis*) is extensive; there is little geographic separation between the numerous species, spawning occurs at the same temperature threshold, and furthermore, spawning sites are limited and similar for most species where nests are commonly exchanged (Avise & Saunders 1984; Konkle & Philipp 1992; Avise 1994).

If reproductive isolation between two species or populations is maintained by geographic separation, and they are brought together by man or natural causes (e.g. stream capture or tectonics (DeMarais *et al.* 1992)), they may hybridize. Indeed, hybrids are often only found in areas of habitat disturbance (Capula 1993; Rhymer & Simberloff 1996), although there are exceptions to this generality (Thoma & Rankin 1988). Hybridization is relatively common among temperate freshwater fishes in areas where geologic and climatic events since the Pleistocene have drastically altered aquatic environments, but hybridization appears to be rare among marine and tropical fishes that inhabit more stable environments (Campton 1987). Man-made habitat changes have been correlated with hybridization between previously allopatric and naturally sympatric pairs of species, particularly when the physical structure of the habitat is altered by damming (Verspoor & Hammar 1991; Balon 1992). The widespread stocking of fishes outside their native geographic ranges for fishery enhancement or other management purposes has frequently resulted in hybridization between native and introduced forms (Hindar *et al.* 1991; Ogutu-Ohwayo & Hecky

1991; Carvalho 1993; Carvalho & Hauser 1995). Hatchery-reared fish frequently hybridize with endemic species due to their intentional release or accidental escape. For example, rainbow trout (*Oncorhynchus mykiss*) repeatedly introduced into endemic cutthroat trout (*Salmo clarki seleniris*) habitat resulted in extensive introgressive hybridization (Busack & Gall 1981; Gyllensten *et al.* 1985). Species of pupfish (*Cyprinodon*) have also commonly hybridized following introductions (Echelle *et al.* 1987; Echelle & Echelle 1994; Childs *et al.* 1996). The ease with which fish hybridize has often been overlooked by, and presents many problems to, fishery biologists and management agencies.

1.3.2 Introgression

The interbreeding of genetically and reproductively distinct populations of fish, where viable hybrid offspring are produced, is potentially an important pathway for genetic exchange both within and between fish species (Mayr 1963; Verspoor & Hammer 1991). Hybrid populations that have progressed past the F_1 stage, with little or no loss on viability or fertility, can be described as genetic (or population) admixtures (Campton 1987). The transfer of genes between taxa, when hybrid progeny are fertile and hybrids back-cross into one or other parental population, may give rise to temporary or permanent changes in the gene pools of the hybridizing populations. Mayr (1963) termed changes which involve the incorporation of novel genes from one species or semi-species into another as introgression, whereas he referred to the transfer of genetic material between populations within species as gene flow. However, a more workable and perhaps more biologically meaningful distinction between introgression and gene flow is to define introgression as "the transfer and incorporation of novel genetic variants into a population by hybridization with another. Gene flow can then be considered to encompass all genetics transfers, including introgression, or be used less globally as the transfer of non-novel variation" (Verspoor & Hammer 1991). The point at which hybridization results in introgression is also difficult to define, since there is no *a priori* basis for deciding at which generation of back-crossing hybrid offspring are to be considered an integral part of one or other parental population and the novel genes they carry can be considered introgressed. A possible operational definition of introgression is the presence in a parental population of back-crossed hybrid individuals with novel genetic variation which are reproductively competent (Verspoor & Hammer 1991).

Introgression can vary along a continuum, as discussed when referring to hybridization, although introgression between taxa can be ruled out *a priori* when hybrids are completely

infertile. The repeated backcrossing of hybrid descendants with a parental species can further result in the introgression of genes from one species into the gene pool of another; a process that can result in the genetic loss of an entire species, subspecies, or unique population (Campton 1987; Rhymer & Simberloff 1996). Selection and genetic drift can result in the loss of introduced variation, although these process, particularly selection, may result in the incorporation of novel genetic variability (Barton & Hewitt 1985). Even when introgression is extremely rare between species, it may still be a more important factor than mutation as a source of new viability within taxa (Anderson 1949). Grant & Grant (1996) estimated that the additive genetic variance introduced by hybridization of Darwin's finches (*Geospiza*), is two to three orders of magnitude greater than that introduced by mutation alone. Introgression can result in the production of individuals with novel genetic make-up and morphology that can provide the starting point of a new evolutionary trajectory (Grant & Grant 1996; Dowling & Secor 1997), particularly if the variation is selectively important and changes the adaptive niche of a population or species (Section 1.3.6). Several studies have provided circumstantial evidence for the existence of introgressed genes which have a selective advantage. For example, the presence of a *mMDH-B* allele at high frequency in a red shiner *Notropis lutrensis* population, a result of introgression from *N. venustus,* strongly suggests that it is affected by selection since the spatial distribution of the *mMDH-B* allele is correlated with temperature (Zimmerman & Richmond 1981). Furthermore, a *MEP-2* allele introgressed into brown trout (*Salmo trutta*) from Atlantic salmon (*S. salar*) in Scotland, is affected by thermal selection (Verspoor & Jordan 1989), and affects growth and maturation (Jordan & Youngson 1991).

1.3.3 Hybridization and genetic asymmetries

Genetic asymmetries frequently attend hybridization and introgression processes, leading to patterns of differential exchange for nuclear and cytoplasmic loci. The ability to study genetic asymmetries, is one of many aspects of hybridization which has been greatly facilitated by the advances in molecular genetic analysis. The mtDNA or cpDNA genotypes normally characteristic of one species can occur against the predominantly nuclear background of another species (cytoplasmic 'capture'), presumably because of current or past introgressive hybridization (e.g. Lamb & Avise 1986; Dowling *et al.* 1989; Dowling & Hoeh 1991). Bernatchez *et al.* (1995) examined a population of brook trout (*Salvelinus fontinalis*) in which all individuals possessed the mitochondrial genome of Arctic char (*S. alphinus*), despite being presently allopatric, illustrating

that introgressive hybridization can have significant long-term effects on genetic composition. Cytoplasmic 'capture', and other differential patterns of introgression can be due to interlocus variation in selection intensity against alleles on heterologous genetic backgrounds, gender-specific fitness differences, differential mating behaviours of the hybridizing taxa or other sources of differential gametic exchange (Avise 1994). Selection against introgression is often presumed to involve reduced fitness in backcross generations caused by disruption of coadapted parental complexes (Section 1.3.4). Alternative modes of gene transfer can differentially influence cytonuclear associations, particularly in plants where a pronounced decoupling of male and female components of gene flow across species is possible due to the two distinct avenues for genetic movement; that of pollen and seeds (e.g. Louisiana irises; Arnold 1992; Arnold *et al.* 1991).

In species with heterogametic females, female hybrids more often show decreased fitness, whereas in species with heterogametic males, male hybrids tend to show more severe reductions in viability or fertility. This empirical generality was first noticed by Haldane (1922; cited in Avise 1994): "When in the F_1 offspring of two different animal races one sex is absent, rare or sterile, that sex is the heterozygous [heterogametic] sex", and is known as Haldane's Rule. Where heterogametic hybrid females are sterile, the mtDNA divergence between hybridizing species can remain high indicating a lack of interspecific cytoplasmic genetic exchange, despite the extensive introgression of nuclear genes via male hybrids (e.g. Tegelström & Gelter 1990). Asymmetry of hybrid fertility, where heterogametic males are sterile and the females remain fertile, has been invoked to explain the apparent ease with which mtDNA appears to cross some species' boundaries (Coyne & Orr 1989; Tegelström & Gelter 1990).

Differential mating behaviours commonly influence the genetic architecture of hybrid zones (e.g. Lamb & Avise 1986) (Section 1.3.5). In the North American sunfish (genus *Lepomis)* there is a strong tendency for hybridizations to take place between parental species differing greatly in abundance, and a tendency for the rare species in the hybrid cross to provide the female parent. The absence of conspecific partners and spawning stimuli for females of rarer species might be important factors in increasing the likelihood of interspecific hybridization (i.e. 'density-dependent mating pattern'; Avise & Saunders 1984). Suspected asymmetrical mating behaviour may be confirmed when hybrids share a common mtDNA genotype, such as the F_1 hybrids of *Lepomis macrochirus* and *L. gibbosus,* which all had *gibbosus*-type mtDNA due to the heterospecific sneaky spawning behaviour of *L. macrochirus* males (Konkle & Philipp 1992).

1.3.4 Hybrid vigour, outbreeding depression and the loss of genetic diversity

Hybrid genotypes (recombinants) constitute a source of novel genetic diversity, that can have increased fitness or new ecological tolerances and thereby the adaptive potential for colonizing environments not occupied by either parental population (Dowling & Secor 1997). Such 'hybrid vigour' (heterosis) usually refers to artificially produced hybrids between domesticated, highly inbred strains (Schonewald-Cox *et al.* 1983). In aquaculture, including the aquaculture of *Oreochromis* species, hybrids are often favoured since heterosis can be observed for growth, food conversion and disease resistance. However, due to hybrid vigour, hybridization can lead to the spread of an introduced species to ecosystems previously unsuitable for them. In Australia, for example, the introduction of two strains of the common carp (*Cyprinus carpio*) gave rise to a new, more vigorous and ecologically more tolerant strain, which became far more widespread and problematic than the parental stocks (Arthington 1991).

Although F_1 hybrids may initially exhibit increased fitness, subsequent generations often display a drop in fitness resulting in variable and uneconomic hybrids (Schonewald-Cox *et al.* 1983). This loss of fitness is due to the disruption of 'coadapted gene complexes'. Genes are said to be coadapted if high fitness depends upon specific interactions between them (Wallace 1968; cited in Carvalho 1993). Introduction of genetic novelty through natural or enforced gene flow may result in a breakdown of coadapted genes and thus an overall reduction of fitness, i.e. 'outbreeding depression' (Templeton 1986). This can be a problem when hybrid vigour in the F_1 generation has resulted in the unchecked use of hybrids as brood material, as realized in the Czechoslovak aquaculture of bighead carp (*Hypophthalmichthys molitrix*) and silver carp (*Aristichthys nobilis*) hybrids (Slechtova *et al.* 1991). The concept of coadapted gene complexes is associated with 'local adaption', a process that increases the frequency of traits which enhance survival or reproductive success of individuals in a particular environment (Carvalho 1993). Hybridization can result in the breakdown of, the often fragile and unique, locally adapted gene pools.

Coadapted gene complexes may be specific to a local population, and over generations may represent a concerted population response to specific environmental conditions. Local adaptations are particularly prevalent among salmonids populations (Taylor 1991). Stocks introduced from contrasting habitats, which through local selective pressures are often genetically distinct, can result in hybrids that exhibit an array of traits maladaptive in either source area. If the introduced fish have been reared in a hatchery, where domestication and selective breeding over many generations can result in a loss of genetic diversity due to inbreeding and genetic drift, the effects

of hybridizations on the genetics of indigenous species or population may be even more severe due to the dilution of locally adapted gene pools and thus a reduction in population performance (Daget & Moreau 1981; Hinder *et al.* 1991; Carvalho 1993; Carvalho & Hauser 1995). Innate differences in behaviour and growth in farmed and hybrid (farmed x native) salmon, threaten native populations through competition and disruption of local adaptations (Einum & Fleming 1997). Hybridization not only has the potential to destroy gene pool integrity (Skaala *et al.* 1990) but can also result in the elimination of unique genotypes of indigenous species or populations (Hayes *et al.* 1996) and thereby cause the loss of genetic variability. The destruction of coadapted gene complexes in local populations is as irreversible as the loss of alleles or a species. Hybridization often means that unique qualities of individual species or populations are lost forever (Daget & Moreau 1981; Dowling & Childs 1992; Carvalho & Hauser 1995).

1.3.5 Hybrid zones

Most hybrid zones, which often appear linear or mosaic, represent a secondary overlap between formally allopatric or parapatric taxa, where progeny of mixed ancestry are produced (Barton & Hewitt 1989). Hybridizing populations exchange genes and yet, unlike the majority of situations already discussed, remain distinct and therefore present no threat to the genetic integrity of the parental populations. Several theories or models have been proposed to explain the persistence and dynamics of hybrid zones. The 'bounded hybrid superiority' model explains hybrid zone stability due to the superior fitness of hybrids in areas of presumed ecological transition (Moore 1977; cited in Avise 1994). Whereas, in the 'dynamic-equilibrium' hypothesis, correlations between habitat and genotype are not predicted, and hybrid zone maintenance is achieved through a balance between dispersal of parental types into the zone and selection against hybrids which exhibit reduced viability or fertility (Barton & Hewitt 1989). With the discovery that zones may sometimes be mosaic in structure (the former models predict a clinal structure), the 'mosaic hybrid zone' model was developed. In this model, where strong correlations between genotype and habitat are predicted, hybrids are not assumed to be more fit than the parental species and a dispersal / selection balance maintains the zone (Harrison & Rand 1989). Arnold (1997), has recently proposed an 'evolutionary novelty' model, in which hybrid fitness is predicted to be variable and to depend on both hybrid genotype and on habitat. Molecular genetic markers have allowed the geographic structure of hybrid zones and the importance of habitat selection to be estimated (Harrison & Bogdanowicz 1997; Rieseberg 1998).

The evolutionary significance of such hybrid zones is an important topic of research in evolutionary biology (Barton & Hewitt 1985, 1989; Rieseberg 1998). Hybrid zones, where there are often amalgamations of independently evolved genomes, may allow evolutionary forces such as recombination and selection easier to study because their effects are magnified. The effects of intergenomic interactions are also exaggerated. Furthermore, since the populations or species involved are genetically different, multiple markers can be uncovered for characterizing the hybrid gene pool (e.g. Baker *et al.* 1989; Patton & Smith 1992; Harrison & Bogdanowicz 1997). By measuring the associations of cytoplasmic genes with nuclear genes and genotypes within a hybrid zone, cytonuclear disequilibria provide insights into levels of gene flow, age of reproductive barriers, directionality of crosses, levels of assortative mating and mechanisms of selection on hybrids (Arnold 1993). In some hybrid zones, patterns of variation for cytoplasmic and nuclear markers are highly concordant (Baker *et al.* 1989), but in several other instances pronounced discordances across loci appear to reflect differing historical patterns of introgression (Section 1.3.3).

1.3.6 Speciation through hybridization

The genetic instability produced through hybridization usually provides an effective isolating barrier between the two populations, but there are mechanisms by which such an unstable hybrid can lead to a new species, different and isolated from both parents (Templeton 1981; Dowling & Secor 1997). Introgressive hybridization, long recognized as being important in the evolution of plants (Anderson 1949; Grant 1981; Arnold 1997), may also play a significant role in the evolution of vertebrates. Stability and evolutionary independence are essential for distinguishing taxa of hybrid origin from instances of ongoing hybridization among taxa (Dowling & Secor 1997). Hybridization can instantaneously produce distinct taxa, either through polyploidization, where crossing with the parental species produces progeny with unbalanced chromosome sets, or through conversion to an essentially all-female (unisexual) mode of reproduction. Essentially all of the 70 known vertebrate unisexual taxa arose through hybridizations between two related sexual species (Avise 1994), such as the gynogenetic live-bearing fish *Poecilia formosa* generated from *Poecilia latipinna* and *P. mexicana* (Avise *et al.* 1991). A few unisexuals carry genomic contributions from more than two sexual ancestors, such as the trihybrid unisexual forms of topminnow, *Poeciliopsis* complex (Quattro *et al.* 1992). Among fishes, allopolyploidy has been hypothesized for three cypriniform families, including the

successful tetraploid catostomids (Ferris 1984; cited in Dowling & Secor 1997).

New taxa may also be formed through introgressive hybridization, although, unlike the previous two mechanisms of hybrid speciation, diversification is not instantaneous. Gene exchange among taxa produces groups of recombinant individuals that may eventually, after a period of geographic isolation where genetic differences evolve that allow coexistence with parental taxa, lead to stable independent lineages (Dowling & Secor 1997). Several examples of stabilized hybrid forms have been suspected from morphological or distributional considerations. However, morphological and molecular analysis have often not provided support for the hybrid origin scenario, or have failed to discriminate among alternative hypothesis (such as ancestral polymorphism, convergence, and past hybridization) (Avise 1994). For example, when examining the proposed hybrid origin of the white shiner, *Luxilus albeolus*, Meagher & Dowling (1991) found that allozyme alleles supposedly contributed by *L. cerasinus*, were also found in local allopatric populations of *L. cornutus*. These findings prevented the discrimination of hybrid origin from convergence of allozymes or shared ancestral polymorphisms (Meagher & Dowling 1991). Difficulty in detecting hybrid forms may also be due to a lack of morphological differentiation, since hybridization that gives rise to a new lineage can produce character homoplasy that might obscure species true history (Jones *et al.* 1995), although this can often be resolved through molecular analysis. For example, mtDNA analysis revealed the tiger salamander subspecies *Ambystoma tigrinum stebbinsi*, originated through hybridization between *A. t. mavortium* and *A. t. nebulosum* (Jones *et al.* 1995). Molecular analysis has provided much support for the postulated hybrid origin of *Gila seminuda* in the Virgin River of western North America, between the roundtail chub (*G. robusta*) and bonytail chub (*G. elegans*) (DeMarais *et al.* 1992). Introgressive hybridization and geographic separation has been suggested to have played a part in the speciation process in the cyprinid genus *Notropis* (Dowling & Brown 1989). Gene exchange among distinctive forms has contributed to existing diversity, supporting the hypothesis that introgressive hybridization can play a significant role in evolution of vertebrates (Dowling & DeMarais 1993; Bullini 1994; Dowling & Secor 1997).

Introgressive hybridization provides additional genetic variation, which can provide the capacity to rapidly respond to environmental change, which may provide hybrid stocks with a selective advantage over parental forms. McElroy & Kornfield (1993), through the study of morphology in experimentally produced hybrids of Lake Malawi rock-dwelling cichlids, proposed that hybridization in these species could result in the production of novel phenotypes with unique

evolutionary potentials. Crapon De Caprona (1986) considered the possible role of hybridization in cichlid fish speciation through a process where hybrid individuals preferentially mate with other hybrids, discriminating against the parental species. As yet, there is no direct evidence in support of hybridization being important in the evolution of cichlids. The existence of stable hybrid derivatives might be relatively common in some groups of fishes, but remain unrecognized due to a lack of detailed molecular studies. The formal taxonomic status of such introgressed forms is controversial and of relevance to the implication of conservation programmes, particularly when the introgressed population is isolated from its parental species by extrinsic barriers to reproduction. Hybridization among *Gila,* that are critically endangered taxa, has been considered primarily as detrimental, but the apparently pervasive influence of natural introgression suggests that conservation strategies should evaluate the impact of hybridization in a new light (Dowling & DeMarais 1993; Bullini 1994; Dowling & Secor 1997).

1.3.7 Behavioural studies of hybridization and reproductive isolation

Behavioural observations under controlled conditions in the laboratory offer many advantages, especially since one factor can be varied at a time. Controlled behavioural studies provide the opportunity, for example, to stage introductions, know the relatedness and recognize individuals, or to alter the habitat, and thereby design tests of particular hypotheses about behaviour (McFarland 1985; Turner 1986). Furthermore, in the case of aquatic species from turbid environments, recording is greatly facilitated in the laboratory (Turner 1986). The behaviour of animals can also be investigated through experiments involving selective interference in the natural environment. The application of molecular markers has greatly facilitated the detection and verification of organisms studied in the field (e.g. Burke & Bruford 1987; Burke *et al.* 1992; Packer *et al.* 1991; Amos *et al.* 1993; Kellogg *et al.* 1995; Parker & Kornfield 1996).

The behaviour of animals can operate as barriers to gene exchange between species, and populations, at all stages of life history (premating and postmating). Behaviour, for example, is involved in the choice of habitat, timing of reproductive activity, meeting of potential mating partners, recognition of partners, act of copulation and in the functional sexual behaviour of offspring. Study of the development and maintenance of behavioural barriers, and of the behavioural mechanisms underlying species recognition, is important in understanding the biological processes of speciation (Butlin & Ritchie 1994; Dall 1997). Behavioural studies of recently diverged species (and their hybrids) provide the opportunity to assess the involvement of

behaviour, at each stage of life history, in speciation. Such studies also emphasize the importance of using behaviour as well as traditional morphological characters to classify organisms (Dall 1997). Behavioural studies allow assessments to be made on the importance of behaviour in maintaining reproductive isolation when the possible effects of other putative RIMs, such as habitat preference, are removed. For example, Price & Boake (1995) in a study of the role of courtship and aggressive interactions in the maintenance of reproductive isolation between two species of *Drosophila*, found that sexual selection against hybrids alone was unlikely to be a sufficient force to reduce gene flow and maintain species distinctions. Studies on congeneric allopatric and sympatric species have shown that ethological RIMs often fail when present in sympatric species (e.g. Baylis 1976a; Rubinoff & Rubinoff 1971; section 1.2.2.1). Behavioural experiments have also been valuable in providing evidence, or not, for the role of reinforcement in the evolution and maintenance of species distinctions (Butlin 1989). Hatfield & Schluter (1996), in an examination of the contribution of sexual selection to reproductive isolation between sympatric species of stickleback (*Gasterosteus aculeatus* complex), found no evidence of hybrid mating disadvantage and therefore no support for the role of reinforcement. Laboratory and field behavioural studies, combined with molecular genetic analysis, have greatly assisted in understanding the dynamics and maintenance of hybrid zones (Barton & Hewitt 1985; Rieseberg 1998).

Many studies have been concerned with evaluating the genetic basis of behaviour. Most behavioural traits appear to be associated with differences in a number of loci and many genes appear to have pleiotropic effects on behaviour. Non-genetic environmental inputs are also likely to have an effect (Danzmann *et al.* 1993b). The study of hybrids can be productive as a first step to demonstrate a genetic basis for a behavioural difference, where two species display a specific behaviour and the hybrid displays are intermediate (e.g. *Drosophila auraria* complex; Tomaru & Oguma 1994). The study of interspecific crosses can help determine if genes influencing behavioural variation within and between populations are involved at the interspecific level (Hoffman 1994). The analyses and detection of genes responsible for mating preference, central to the theory of sexual selection and speciation processes, has proven to be a difficult task (Ritchie 1992). This is largely because of the practical difficulty of demonstrating the fitness consequences and heritability of preferences, and genetic variability in the chosen traits (Abt & Reyer 1993). Majerus *et al.* (1982; cited in Ritchie 1992) selected lines based on female mate preference between two forms of the two-spot ladybird (*Adalia bipunctata*), and illustrated that mating preference has a genetic component which can be changed by artificial selection, however the replication of these

results has been unsuccessful.

Interest in the behaviour of cichlids has greatly increased in the last four decades, probably because of their commercial importance (as a food source and aquarium fish) and concerns about their conservation. In 1950 the pioneering study by G. P. Baerends and J. M. Baerends-van Roon appeared; *An introduction to the study of the ethology of cichlid fishes*, containing descriptions of the morphology and behaviour of several cichlid species, and some experimental analyses of social behaviour. Lowe-McConnell (1956, 1959) pioneered studies into the reproductive behaviour of tilapia, furthered by the comprehensive taxonomic work of Trewavas (1983). Many ethological studies on cichlid fishes have been carried out in the laboratory, especially those concerning communication and parental roles. Comparisons between field and laboratory behaviour have also been made (Rothbard 1979; Schwanck 1989; Robinson & Turner 1990). Laboratory observations on the reproductive behaviour of cichlids have contributed to the formulation of many classical ethological concepts, such as drives, fixed action patterns, the conflict hypothesis for the evolution of displays, and hierarchical motivational systems (Turner *et al.* 1989). The study of behavioural mechanisms underlying species recognition is crucial to understanding cichlid speciation (Crapon de Caprona 1986). The principal drawback of laboratory studies is the danger of inducing unnatural behaviour. To obviate this, spacious accommodation should be provided and the species chosen should be kept in a social environment which is likely to occur in nature (Robinson & Turner 1990). However, laboratory studies allow the detailed analysis of behaviour in situations where the signals are easy to quantify and the context and communication function can be interpreted (Baylis 1976 b).

Other applications of behavioural studies, which are of concern to the present study, include the insight which can be gained into the likely outcome of fish species introductions (Pitcher 1995).When considering an introduction it is necessary to assess both the impact an introduced species can have on the endemic fauna and flora, and the suitability of the habitat available. One important factor, for example, is the presence and extent of suitable substrata for spawning (Turner *et al.* 1991). Knowledge of reproductive behaviour also has several implications for the design of strategies for species exploitation and conservation (Turner & Robinson *unpubl.*). In *Oreochromis* species, for example, trawling and seining may have detrimental effects by destroying nests and killing breeding fish, and small-meshed beach seines in shallow water may exploit fry. Furthermore, changes in lake level where water has been drawn from reservoirs for hydroelectric generation, can strand nests and effect recruitment due to the elimination of shallow nursery areas.

1.4 METHODS OF DETECTING HYBRIDIZATION

1.4.1 Morphology

Prior to the development of molecular genetic markers (1970s), comparing morphological characters was essentially the only method available for detecting hybridization. In such studies, meristic and morphometric characters are counted and measured on the suspected hybrid individuals and on individuals representing the two hypothesized parental species (Elder *et al.* 1971; Campton 1987). Hybrid individuals are diagnosed if they have, on the average, intermediate meristic counts and morphometric measurements relative to those for the parental species. Results can be verified by artificial matings between the parental species (e.g. Leary *et al.* 1985; Reist & Crossman 1987; Pitts *et al.* 1997). Morphometric analysis has been used to distinguish between the parents and both hybrids where fingerlings (*Cynoscion* species) were stocked together (Procarione *et al.* 1988).

Intermediate characters in hybrids can provide evidence for the genetic control of morphometric traits (Reist & Crossman 1987; Economidis & Sinis 1988), although studies can be complicated where phenotypic variation in morphological characters is subject to environmental modification. Morphological traits are usually polygenic, representing the phenotypic expression of a large number of genes and influenced to varying degrees by environmental effects (Van der Bank & Ferreira 1987; Beacham 1990; Robinson & Wilson 1995). It is therefore difficult to precisely know, for an individual or species, the full range of interspecific phenotypic variation possible for morphological traits (Leary *et al.* 1983, 1985). The technique is also ineffective in studies where there is very little phenotypic differences, or an overlapping of characters, between the parental species concerned (McAndrew & Majumdar 1983; Slechtova *et al.* 1991).

Morphological characters used to distinguish parental species are usually not uniformly intermediate in known or suspected hybrids (Leary *et al.* 1983, 1985). The hybrid may appear morphologically intermediate for all characters combined, but for a specific character it may closely resemble one of the parental species (Emery & Chinnappa 1992; McElroy & Kornfield 1993). Morphological data can provide only circumstantial evidence for hybridization because hybrids are usually assumed *a priori* to be morphologically intermediate to the parental species. Furthermore, purity in individuals representing the parental species has to be assumed. The extent of hybridization or introgression cannot be determined from morphological data since F_1, F_2 or backcross hybrids may not be individually distinguishable. Detecting introgression may also be extremely difficult, and therefore underestimated, because introgressed populations may appear

morphologically identical to one of the parental species (Elder *et al.* 1971; Busack & Gall 1981; Katoh & Ribi 1996). Despite these downfalls, morphological characters are valuable for the initial detection of putative hybrids, which can then be confirmed using molecular markers (Cesaroni *et al.* 1992; Emery & Chinnappa 1992; Brown 1995).

1.4.2 Molecular genetic markers

Molecular genetic markers have revolutionised the ability of scientists to analyse organismal relationships and diversity, which previously relied largely upon comparisons of phenotypic features. Molecular genetic markers have been used to address numerous aspects of the behaviours, life histories, and systematics of organisms (Hillis & Moritz 1990; Burke *et al.* 1992; Avise 1994; Dhondt 1996). In fisheries and aquaculture the application of molecular techniques have proven to be extremely valuable, especially in studies of stock structure, genetic variability, species identification and hybridization (Utter 1990; Wright 1993; Ferguson 1994; Park & Moran 1994; Ferguson *et al.* 1995; O'Connell & Wright 1997; Carvalho & Hauser 1994, 1998). The ability to analyse genes directly in hybridizing populations, allows for an accurate assessment of the actual extent of introgressive hybridization among populations. The techniques available include karyotyping, allozyme electrophoresis, and more recently, mitochondrial DNA analysis and nuclear DNA analysis. Cytological analysis has proven of limited use in fishes due to a lack of detectable karyotypic variation, and the difficulty of working with the large numbers of small chromosomes present in most fish species (Campton 1987). Ribosomal DNA has also been used to some extent for the analysis of genetic introgression across hybrid zones (Baker *et al.* 1989). Here, the molecular genetic techniques most widely used in detecting hybridization; allozyme electrophoresis, nuclear DNA analysis (particularly the 'RAPD' technique) and mitochondrial DNA analysis will be described. Although the last technique was not used in the present study, its popular use in detecting hybridization and introgression warrants a brief description.

1.4.2.1 Allozyme electrophoresis

Allozyme analysis provides genetic markers, which are codominant and inherited in a Mendelian fashion, by the electrophoretic separation of proteins through a starch, cellulose acetate or acrylamide gel. Migration of the protein is determined by its charge, size and shape (which in turn depend on the amino acid sequence of the protein), and the pH of the running conditions. The proteins amino acid sequence is determined by the nucleotide sequence of the coding gene,

thereby, allowing the indirect survey of polymorphisms in the DNA sequence.

Allozyme analysis has been valuable in the management of cultured fish in breeding programmes (Moav *et al.* 1976), and in detecting the genetic impact of culture by providing evidence of genetic change or the loss of genetic variability in aquaculture stocks (Ryman & Utter 1987; Utter 1990; Ferguson 1980). However, since the vast majority of nucleotide substitutions has no effect on gene products, allozyme electrophoresis significantly underestimates the amount of variation present within a taxon and has sometimes largely failed to detect polymorphisms (e.g. Kornfield 1991). The indirect approach of protein analysis to detect DNA polymorphisms results in other constraints, especially when applied to systematic studies, although these are not such a concern when using the technique to detect hybridization. It is not possible to estimate genetic distance among alleles because two alleles are either identical or different, therefore, population analysis of allozyme data is based entirely on genotype and allele frequencies. Furthermore, there is some controversy on the neutrality of allozyme markers, that is, whether polymorphisms at any one locus are products of neutral mutations or whether they are selectively maintained (Ward *et al.* 1992; Ferguson 1994). Any meaningful application of allozyme analysis demands the ability to correctly interpret gels. Satellite bands, overlapping loci and environmental induction can complicate, or lead to incorrect interpretations of banding patterns and, in turn, of the detected polymorphisms (Richardson *et al.* 1986). Breeding studies and, more commonly, conformity of banding patterns with expectations based on molecular structure of the enzyme provide evidence for genetic differences underlying the allozyme polymorphisms. Another problem is that slight differences in electrophoresis conditions may cause different relative mobilities, often complicating the comparisons of results from different laboratories (White & Shaklee 1991).

Detecting hybridization and introgression by electrophoretic methods is relatively straightforward, especially, when the two parental species are fixed for different alleles at two or more loci (Douglas *et al.* 1995; Katoh & Ribi 1996). In these circumstances, F_1 hybrids will be heterozygous for the different parental alleles at all diagnostic loci. If hybridization has proceeded past the F_1 stage, indicating hybrid fertility, hybrid descendants will express a broad mixture of recombinant types, including the two parental types (Macaranas 1986; Carmichael *et al.* 1993). A minimum requirement for the identification of all post-F_1 hybrids is two unlinked diagnostic loci, although the discrimination of almost all F_1 from post-F_1 fish is only possible when using six or more independent diagnostic loci (Campton 1990). Separation of different hybrid generations, beyond distinguishing F_1 and post-F_1 individuals, is not feasible as any parental or hybrid genotype

combination is possible in any generation beyond the F_1. If alternative alleles exist in the parental species, patterns of introgression can be quantified and asymmetries in gene flow may be revealed (Cesaroni *et al.* 1992). Allozymes can also be used to mark F_1 hybrids between species, so that differences in growth rate and developmental rate may be compared between the parental and hybrid stocks when they are reared in a common environment (Danzmann *et al.* 1993). Furthermore, the predicted reduction in fitness of hybrids between distinct gene pools has been documented using allozymes (Philipp 1991).

Detecting hybridization is more difficult if the two species are not fixed for different alleles at one or more loci, but it is possible if they have different common alleles (Campton & Utter 1985). Hybridization can only be investigated quantitatively by determining whether the proportion of fish with intermediate genotypic combinations is significantly greater than one would expect from the random mating within one or both species (Campton 1987, 1990). This approach is particularly useful for detecting hybridization between taxa below the species level where fixed allelic differences are often absent. The more loci used and the greater the allele frequency differences at each locus, the greater the power of this approach to detect hybrids (Verspoor & Hammer 1991). Due to its ease, speed and relatively low cost, allozyme electrophoresis remains a popular technique for the detection of hybridization, particularly when combined with mitochondrial DNA analysis (Friesen *et al.* 1993; Gyllensten *et al.* 1985).

1.4.2.2 Mitochondrial DNA analysis

Mitochondrial DNA (mtDNA) is a small molecule, about 15-20 kb in length, and since it is extranuclear and inherited via the maternal cytoplasm, provides genetic information independent of nuclear genes. MtDNA also differs from nuclear DNA in its organisation and the speed and mode of its evolution (Harrison 1989). The mtDNA genome is highly efficient containing far fewer non-coding and repeat sequences than nuclear DNA (Meyer 1993). The predominantly clonal and maternal inheritance of mtDNA (see Fisher & Skibinski 1990, and Magoulas & Zouros 1993, for exceptions) reduce the effective population size to one-fourth of that for nuclear DNA of the same organism (Nei & Tajima 1981), therefore making it more sensitive to stochastic evolutionary processes, and thus particularly useful for the detection of population differentiation and for phylogenetic studies (Ferguson 1980). The level of population differentiation detectable with mtDNA is often not detected using conventional electrophoretic analysis because of high genetic similarity among populations. For example, fish stocks can be discriminated with mtDNA analysis

whereas stocks are usually not fixed for different allozyme alleles (Danzmann *et al.* 1993a), although the converse situation is sometimes seen (e.g. Turan *et al.* 1998).

The application of the polymerase chain reaction (PCR) to mtDNA studies obviates the need to isolate mtDNA by extended ultracentrifugation and perform conventional cloning, thus allowing the analysis of more and larger samples and also a greater chance of detecting polymorphisms (Saiki *et al.* 1988; Kocher *et al.* 1989). In the PCR a thermostable DNA polymerase is used to copy cyclically the sequence between two priming sites on opposite DNA strands; the reaction requires the presence of two primers complimentary to these sites and the amount of product doubles with every cycle (Saiki *et al.* 1988). The amplified region can then be analysed either by RFLPs (restriction fragment length polymorphism) or by direct sequencing.

Restriction analysis of mtDNA involves the digestion of purified DNA with a series of enzymes which cleave the DNA when short specific nucleotide sequences are encountered; the fragments are separated by electrophoresis and visualised directly or indirectly with radio isotopes. It is relatively easy to isolate mtDNA and perform restriction analysis; the use of restriction endocleases has revealed extensive intraspecific and interspecific sequence variation in mtDNA of mammals, reptiles and fish (e.g. Gyllensten *et al.* 1985; Bembo *et al.* 1994; Ruzzante *et al.* 1996; Clifford *et al.* 1998). However, there are number of limitations to the techniques, for example, since mtDNA is maternally inherited results may not be congruent with those from nuclear analysis, and restriction analysis only detects a portion of the variation present in the mtDNA molecule (Kornfield 1991).

The most useful properties of mtDNA for studying hybridization are its predominantly maternal inheritance as a clonal genetic marker and the absence of recombination. The maternal species can therefore be identified in known or suspected F_1 hybrids if one of the two parental species are characterised by different mtDNAs (Friesen *et al.* 1993; Karl *et al.* 1995). However, since mtDNA can only identify the maternal species, the identification of hybrids is only possible when combined with another technique. Mitochondrial DNA techniques, when used with other molecular genetic markers, can detect the introgression of mtDNA, and its direction, from one species into the nuclear background of another species (e.g. Dowling *et al.* 1989; Carr & Hughes 1993; Duvernell & Aspinwall 1995; Childs *et al.* 1996). The combined use of molecular markers can also reveal asymmetries in mate choice or reproductive isolation between hybridizing taxa (e.g. Konkle & Philipp 1992; Arnold 1993; Patton & Smith 1993; Taylor & Hebert 1993; Quesada *et al.* 1995), and provide a genetic tag to monitor the survivorship and competitive ability of hybrids

(Danzmann *et al.* 1993b). Furthermore, historical hybridization events can be detected when individuals of a particular species, as identified by other methods, possess the mtDNA characteristic of a closely related species (Duvernell & Aspinwall 1995; Bernatchez *et al.* 1995).

1.4.2.3 Nuclear DNA analysis

The development of improved cloning techniques and of PCR techniques has allowed the exploitation of the virtually unlimited source of genetic variability present in nuclear DNA. The ability to obtain DNA by non-destructive methods, such as from fin tissue, and the relatively small quantities of DNA required for PCR, have also aided the development and application of DNA markers. For example, it is now possible to analyse historic samples from museums, scale bones and even smoked salmon (Pendas *et al.* 1995). Genomic DNA markers have predominantly been produced through the examination of random or specific regions of single copy nuclear DNA (scnDNA) and repetitive DNA sequences (mini- and microsatellites).

Restriction analyses of scnDNA traditionally relied on Southern blotting procedures (e.g. Wirgin *et al.* 1992). The generation of scnRFLPs has been improved with the use of the PCR technique, and has also been applied to the generation of species-specific markers and thereby to the identification of hybrids (Gross *et al.* 1996). However, due to the considerable effort and expense required for the development of probes and primers, there have been rather few applications of scnRFLP approaches in population genetics and evolutionary biology, despite their widespread application in the mapping of disease genes and quantitative trait loci (Avise 1994). The development of random amplified polymorphic DNA (RAPD) avoids the need to design specific primers and therefore removes the need, and effort required, to obtain prior sequence information (section 1.4.2.4).

The discovery of the high variation in the number of repeat units among individual genomes (Jeffreys *et al.* 1985) has lead to the development of new approaches for the direct investigation of nuclear DNA (Wright 1993). These 'variable number of tandem repeats' (VNTRs) are classified depending on the length of the repeat sequence, as minisatellites (10-40 bp) or microsatellites (2-10 bp), which also differ in their distribution in the genome, their rate and mode of mutation, and their analysis. Length variation in tandemly-arrayed repetitive DNA is usually due to increases or decreases in the repeat copy unit number (O'Reilly & Wright 1995). In multilocus DNA profiling, length variation is surveyed at many VNTR loci simultaneously. The procedure involves the digestion of genomic DNA with a restriction endonuclease, that cuts the sequence

flanking a minisatellite locus, followed by the use of a repeat sequence as a probe in Southern blots at low stringency. Variation in the number of repeats causes differences in the size of the restriction fragments, revealed in a complex multi-fragment pattern ('DNA-fingerprint') that is usually highly informative and individual-specific.

Although not generally applied to the detection of hybridization, multilocus DNA profiling has proved extremely useful for behavioural studies. Multilocus probes are usually applicable over a wide range of species, and have been valuable in the analyses of mating systems through parentage testing (Burke & Bruford 1987; Burke 1989) and estimations of the degree of relatedness among members of cooperatively breeding groups (Burke *et al.* 1989; Amos *et al.* 1993; Packer *et al.* 1991). Other applications have included the assessment of the levels of inbreeding in cultured fish populations (Harris *et al.* 1991; Wright 1993), the genomic selection for introgression breeding programmes (Hillel *et al.* 1990), and the determination of genetic relatedness within fish shoals with a view to the evolution of social behaviour and the possible implications to fisheries (Carvalho *et al.* 1994). However, due to the complexity of multilocus banding patterns it is impossible to assign allele frequencies, therefore restricting the applicability of the approach to population studies. Multilocus DNA fingerprinting is also laborious and requires radioisotopes and large quantities of DNA (O'Reilly & Wright 1995).

An alternative approach, using single-locus probes, has overcome many of the shortcomings of multilocus profiling. Allelic variation is surveyed at individual VNTR loci through Southern blotting procedures, similar to multilocus profiling but with more stringent hybridization conditions and a specific VNTR locus probe (Bentzen *et al.* 1991), or through PCR-based methods where the locus is amplified using primers flanking the array (McGregor *et al.* 1995; O'Connell & Wright 1997). Band profiles are far less complicated and, since bands can be ascribed to a given locus, allele frequencies can be estimated. However, probes are often highly species-specific so a marker system has to be developed in each species of interest (Avise 1994), and in population applications it is difficult to estimate the possible range of allele sizes likely to be encountered which may not be amplifiable with conventional PCR (O'Reilly & Wright 1995).

The shorter VNTR units, microsatellites, are usually analysed by PCR amplification. Primers are shorter and therefore easier to clone and sequence than those for minisatellite analyses, and often amplify unrelated species (Burke *et al.* 1992; Rico *et al.* 1996). Microsatellite markers are codominant and inherited in a Mendelian fashion. The high mutation rate and consequently high levels of polymorphism of microsatellite loci make them particularly useful in situations

where there is little variability at other marker systems or in highly inbred lines (Wright & Bentzen 1994). Microsatellites are useful in kinship and sociobiology studies (Kellogg *et al.* 1995; Parker & Kornfield 1996), or where genetic differentiation may be limited, as in recently diverged or geographically proximate populations (Wright & Bentzen 1994) and have been used in the genetic examination of hybrid zones (Abernathy 1994). However, data analysis in many of these applications is hindered by a lack of knowledge of the nature of mutations at microsatellite loci, and the requirement for prior sequence information (Goodier & Davidson 1993), though some loci may be highly conserved among species (e.g. Rico *et al.* 1996; O'Connell & Wright 1997).

1.4.2.4 Random Amplified Polymorphic DNA (RAPD)

'Random Amplified Polymorphic DNA' (RAPD), as described by Williams *et al.* (1990), is the amplification of random DNA segments by PCR with single primers of short (10 bp long) arbitrary nucleotide sequence, under low annealing temperatures. Through the use of random primer sequences, the application of the RAPD technique requires no previous knowledge about gene sequences in the target species and is therefore suitable for work on anonymous genomes. Two modifications of detecting RAPD markers have been described: 'Arbitrarily Primed Polymerase Chain Reaction' (AP-PCR) developed independently by Welsh & McClelland (1990) using slightly longer primers (10-15 bp) under different amplification conditions to RAPD, and 'DNA Amplification Fingerprinting' (DAF) using shorter random primers of 5-8 bp which produce more complex DNA profiles (Caetano-Anollés *et al.* 1991).

In RAPD analysis, unlike standard PCR conditions, only a single primer is employed. During PCR the random oligonucleotide primers anneal to several priming sites on the complimentary sequences in the template DNA. If two priming sites are within an amplifiable distance, the intervening region will be amplified. The amplification products are resolved on agarose gels or, to visualize a greater number of the amplification products, on polyacrylamide gels (Bardakci 1996). The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Variation is caused by point mutations within priming sites and large additions or deletions (Dowling *et al.* 1996), which affect the efficiency of amplification and change fragment sizes, resulting in the presence and absence of bands (i.e. RAPD markers are dominant). It is, therefore, not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. Amplification of non-nuclear RAPD markers is negligible because

of the relatively small non-nuclear genome sizes (Hadrys *et al.* 1992).

As well as overcoming the need for prior DNA sequence information, the RAPD technique has the potential to sample the genome more randomly than conventional methods since primers of random sequence do not discriminate between coding and non-coding regions (Lynch & Milligan 1994). Other advantages of the RAPD technology include its efficiency, relatively low expense, and the ability to process many samples simultaneously (Haig *et al.* 1996). The speed and efficiency of RAPD analysis has allowed the making of high density genetic maps in many species including plant (e.g. faba bean, *Vicia faba* (Torres *et al.* 1993); apple (Hemmat *et al.* 1994)) and fish species (e.g. tilapia, *O. niloticus* and *O. aureus* (Naish *et al.* 1995a), rainbow trout; *Oncorhynchus mykiss* (Jackson *et al.* 1995)). Unlike many other nuclear DNA markers, RAPD avoids the use of radioisotopes because amplified products are directly visualised (Hadrys *et al.* 1992). As with conventional PCR techniques, RAPD is applicable to problems where only limited quantities of DNA are available, such as for the analyses of museum specimens and rare fish using DNA isolated from scales and fin clippings without destroying the whole organism (Hadrys *et al.* 1992). Amplification with RAPD primers is extremely sensitive to single-base changes in the primer-target site, suggesting that RAPDs should be useful for phylogenetic analysis among closely related individuals (Williams *et al.* 1993).

There have been numerous applications of RAPD in molecular ecology because the specificity of any single RAPD marker may range from the level of the individual to higher taxonomic levels (Hadrys *et al.* 1992). RAPD markers have been applied to parentage analysis (e.g. Lewis & Snow 1992; Ahmad *et al.* 1996), thereby allowing assessments of reproductive success, however, the presence of non-parental bands in offspring of known pedigrees has caused concerns over the suitability of this method for determining parentage (Scott *et al.* 1992; Scott & Williams 1993; Reidy *et al.* 1992). The detection of individuals, and indeed of hybrid populations, relies upon the identification of diagnostic RAPD markers for the parental genotypes under investigation (Marshall *et al.* 1994; Dawson *et al.* 1996; Khasa & Danik 1996). RAPD makers have proven powerful enough for the identification and differentiation of closely related species and conspecific populations, such as mosquito (Kambhampati *et al.* 1992); parasitic protozoa (Tibayrenc *et al.* 1993) and tilapia (Bardakci & Skibinski 1994; Dinesh *et al.* 1996), and for the measurement of genetic variation between cultivated strains of *Oreochromis niloticus* (Naish *et al.* 1995b). Species-specific markers, generated by RAPD, have the ability to demonstrate interspecific gene flow across hybrid zones (e.g. iris (Arnold *et al.* 1991); fire ants (Shoemaker *et al.* 1994); eucalyptus

(Sale *et al.* 1996)) and analyse other hybridization events where allozyme studies have not proven sufficiently sensitive for detection of hybrid genotypes (Quiros *et al.* 1991).

Diagnostic RAPD markers can be eluted from the gel, reamplified, radiolabelled with ^{32}P and used as an inexhaustible supply of probe in Southern analyses (Hadrys *et al.* 1992). Probes have been used for the identification of microorganisms, and are particularly useful when the number of the individuals concerned is very low (Fani *et al.* 1993). The specificity of the probes can be further improved by sub-cloning and sequencing the reamplified diagnostic band, and eventually selecting a partial consensus sequence as a probe (Hadrys *et al.* 1992). Alternatively, a pair of primers can be designed that specifically amplify the region of interest. The RAPD technique has allowed the identification of markers linked to traits of interest without necessity for mapping the entire genome. Such sequence characterized amplified regions (SCARs) have been developed to detect disease resistance genes (Paran & Michelmore 1993; Adam-Blondon *et al.* 1994) and generate species-specific markers (Cargill *et al.* 1995; Bardakci 1996). SCARs are PCR-based markers that represent single, genetically defined loci that are identified by PCR amplification of genomic DNA with pairs of specific oligonucleotide primers. SCAR markers can be advantageous over RAPD markers as they detect a single locus, their amplification is less sensitive to reaction conditions and they can potentially be converted into codominant markers (Paran & Michelmore 1993). SCARs can also be derived from cloned sequences such as RFLP probes, but may not be as useful as PCR-based genetic markers (see Paran & Michelmore 1993).

As with other molecular genetic techniques, the number of scorable polymorphisms can be increased by using single-strand conformation polymorphism (SSCP) analysis. Two DNA strands from the same PCR product often run in different places on SSCP gels and some PCR products from identical places in the two parental genomes may have internal sequence polymorphisms that will resolve as mobility differences on a SSCP gel (McClelland *et al.* 1994). McClelland *et al.* (1994) found the rate of detection of polymorphisms from arbitrarily primed PCR experiments on recombinant inbred mouse genomes, was 20% greater using SSCP gels than using denaturing gels.

The ability of the RAPD technique to reveal intraspecific variation can be used for a variety of population genetic studies, such as screening for the degree of inbreeding (Dinesh *et al.* 1996). However, the analysis of population structure using RAPD has been complicated and hampered by the dominant detection of RAPD markers, which makes the identification of heterozygotes impossible (Lynch & Milligan 1994). The lack of complete genotypic information enhances the sampling variance associated with single loci as well as inducing bias in parameter estimation

(Grosberg *et al.* 1996). Other disadvantages of the RAPD technique involve problems associated with the reproducibility of banding patterns, as slight variations in the reaction conditions, such as template quality or polymerase activity, may affect the amplification of specific fragments (Micheli *et al.* 1994). Only strictly standardized reaction conditions will guarantee reproducible amplification products (Hadrys *et al.* 1992; Black 1993; Cobb & Clarkson 1994; Mailer *et al.* 1994). Furthermore, as with multilocus profiling, where multiple markers appear on the same gel, there can be uncertainty in assigning markers to specific loci in the absence of a preliminary pedigree analysis (Allegrucci *et al.* 1995; Grosberg *et al.* 1996).

'Amplified Fragment Length Polymorphisms' (AFLP), a technique recently developed by Vos *et al.* (1995), which is based on the selective amplification of restricted fragments from digested genomic DNA, attempts to combine the favourable attributes of both RFLP and arbitrary primer methods. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites (Vos *et al.* 1995). The AFLP technique appears to be more repeatable between laboratories than other arbitrary primer methods because reaction conditions used for primer annealing are more stringent (Jones *et al.* 1997).

1.5 THE BIOLOGY AND AQUACULTURE OF TILAPIA

The Tilapiine cichlids originate solely from the African continent and from Palestine, although many artificial introductions have been made within and outside their native range, and they can now be found in most tropical and subtropical countries of the world (Lowe-McConnell 1959; Trewavas 1983; Pullin *et al.* 1997). Some species are specialized and have restricted distributions, while others have broad ecological tolerances and are widely distributed. They have colonized highly diverse habitats including permanent and temporary rivers, deep lakes, swampy lakes, hot springs, lagoons and brackish lakes (Lowe-McConnell 1959; Trewavas 1983). Due to the ease with which tilapia can be bred in captivity and the wide variety of water conditions in which they will grow, tilapia are of great economic importance and provide a readily available source of protein.

1.5.1 Classification and phylogeny

All the Tilapiine cichlids were initially included in the genus *Tilapia* (Boulenger 1899; cited in Trewavas 1983). Regan (1920; cited in Trewavas 1983) revised this original classification

by separating the *Tilapia* group from *Haplochromis*, and dividing *Tilapia* into four subgenera on the basis of ostelogical features. Thys van den Audenaerde (1968) classified the species into the genus *Tilapia* and the subgenera of *Sarotherodon* and *Oreochromis*. Peters & Berns (1978, 1982; cited in Sodsuk & McAndrew 1991), based on their evolutionary ideas of the group, believe that any subdivisions are not justified and that the various forms should all be called *Tilapia*. 'Tilapia' is still used as a common name by both scientists and culturists. However, the most widely accepted classification is that of Trewavas (1982, 1983) where the tilapia are divided into three genera, based primarily on the mode of parental care over eggs and young; the substrate spawning *Tilapia*, the biparental mouthbrooding *Sarotherodon*, and the maternal mouthbrooding *Oreochromis*. Classification is also based on their development, feeding habit, structural features and biogeography. *Oreochromis* species are mainly endemic to East Africa, whereas *Sarotherodon* and *Tilapia* are found predominantly in West Africa.

Male reproductive colouration is also regarded as an important taxonomic characteristic in the family Cichlidae. For example, Trewavas (1983) regarded distinctive territorial colouration as maintaining a degree of reproductive isolation and treated the respective populations as species, thereby separating *Oreochromis mortimeri* from *O. mossambicus*, and *Oreochromis niloticus* into a number subspecies. Trewavas (1983) also assigned the Lake Albert population of *Oreochromis niloticus* to the subspecies *O. niloticus eudarianus* rather than *O. n. niloticus*. However, observations by Turner (1988) have indicated that much, if not all, of such colour variation between *O. n. niloticus* and *O. n. eudarianus* could possibly be due to environmental factors, and therefore stressed that care must be exercised in the use of colour patterns in taxonomic studies.

In addition to the identification of reliable taxonomic characters, there has been much debate on the phylogenetic relationships of tilapia. It is generally agreed that mouthbrooders evolved from a substrate spawning ancestor (McAndrew & Majumdar 1981). Peters & Berns (1978, 1982) suggested that mouthbrooders have diverged many times from substrate spawners, and the time since this divergence occurred has determined the state of advancement of mouthbrooding characteristics. The maternal mouthbrooders were the first to diverge and the paternal and biparental were the most recent to diverge. According to Trewavas (1980) the mouthbrooders, already diverged from the ancestral group of substrate spawners, quickly divided into the group *Sarotherodon* which remained conservative regarding breeding behaviour, and a more progressive group which led to the *Oreochromis*. Electrophoretic studies of the three genera by McAndrew & Majumdar (1981) failed to decide between the two hypotheses, although the close

similarity of the maternal mouthbrooders to each other and the closer relationship of the *Sarotherodon* species to the *Oreochromis* species than to the *Tilapia*, did not favour the hypothesis of Peters & Berns (1982). A more comprehensive study by Sodsuk & Majumdar (1991) concluded that the classification of Trewavas (1983), that divides the group into three genera, is in concordance with their results. Other taxonomic studies (Pouyaud & Agnése 1995) have largely conformed to the taxonomy proposed by Trewavas (1983).

1.5.2 Reproductive behaviour

The alternative breeding strategies of the three tilapiine genera (*Tilapia*, *Oreochromis* and *Sarotherodon*) greatly influences their respective reproductive behaviour. The breeding biology of the tilapia exhibits great plasticity, exemplified by the variation within and between species in, including, parental roles, mating systems, reproductive seasonality and size at maturity. Plasticity is often evolutionary advantageous, as it allows flexible responses to variations in environmental and ecological conditions.

The maternal mouthbrooder (*Oreochromis*) species show a marked sexual dichromatism where the males, who compete for females, are the more highly coloured sex. Other sexually selected characteristics of males are their larger size and more aggressive behaviour than females, the development of secondary sexual structures (e.g. dorsal fin extensions, genital papillae and exaggerated jaws), and the building of nests. *Sarotherodon* species, where both sexes or just the male mouthbroods the eggs and young, usually have minimal sexual dichromatism. Similarly, there is little sexual dimorphism in *Tilapia*, the biparental caring substrate spawners, although the male is generally larger than the female. Both sexes develop breeding colours and their parental roles are similar with both assisting in territory formation and nest building.

In many parts of the tropics, tilapiine cichlids appear to breed all year round, although breeding is often triggered by the rainy season or rising temperatures (Lowe-McConnell 1959; Trewavas 1983). Breeding season often overlaps between species, possibly due to a common response to environmental triggers (e.g. Turner *et al.* 1991b).

1.5.2.1 Tilapia

In the *Tilapia* a relatively long period of pair-bonding occurs before spawning, and can occur before territory establishment. Prior to pairing, both male and female *T. mariae* fight with conspecifics forming dominance hierarchies which, at least in some degree, determine access to

mates (Turner *et al.* 1989a). After several hours to some days of courtship the couple is formed. The monogamous pair spawn repeatedly, alternately applying their genital papilla to the bottom depositing a batch of eggs which are then covered with milt. The eggs adhere to the substrate and are guarded by both parents until the young are able to fend for themselves. The female spends most time fanning the eggs with her pectoral fins, while the male defends the territory and offspring from intruders. *T. mariae* males may occasionally practise simultaneous polygyny and may also desert their mates during the parental period (Schwanck 1989). The mobile young hatch 2-3 days after spawning and remain adhered to the substrate ventilating with their tails ('wriggler phase'). Upon swimming, the fry aggregate and stay in the immediate vicinity of the nest.

1.5.2.2 Sarotherodon

Biparental mouthbrooders often form a stable couple. Both sexes, in biparental and paternal mouthbrooders, co-operate in preparing and defending the spawning site (Fryer & Iles 1972). Exclusively paternal care is only known for *Sarotherodon melanotheron* where limited field observations indicate that males nest colonially (Trewavas, 1983). Aquarium experiments by Balshine-Earn & McAndrew (1995) confirmed that in *S. melanotheron*, where females should have greater potential reproductive rate and thus compete for males, the females were more aggressive and initiated mouth fights more often than males. Also, as expected, males prefer to mate with larger females, while females do not choose mates on the basis of size. This is precisely the reverse of the roles shown by maternal mouthbrooders, such as *Oreochromis*, where females are choosy (Balshine-Earn & McAndrew 1995). Both male and female *S. galilaeus* prefer to mate with larger partners, although females exert a stronger preference. *S. galilaeus,* initially thought to be a monogamous biparental mouthbrooder, appears to represent an intermediate situation where the male and female pair up briefly to dig the nest and spawn, after which each takes eggs into its mouth and goes a separate way (Trewavas 1983).

1.5.2.3 Oreochromis

The breeding behaviour of *Oreochromis* can be seen as a more advanced condition than in *Tilapia*, since it involves a higher degree of ritualization where the courtship process is greatly reduced. For example, in *O. macrochir* it can take less than one minute for courtship, egg laying, fertilisation and batch collection to be complete (Fryer & Iles 1972). Male *Oreochromis* are highly polygynous and may spawn with several females in quick succession; no pair-bonding occurs.

The *Oreochromis* male delimits and defends a territory where it makes a nest to attract and

retain a female. In the aquarium, males begin to develop breeding colouration prior to the establishment of the territory (Baerends & Baerends-van Roon 1950; Neil 1964). Territories are established and competed for through head-to-head threat displays and circling fights. Generally the largest, best-motivated and most aggressive males occupy the best territories, while other males occupy the edges or outside the optimal area (Turner 1986). The central focus of the territory is the 'spawning pit' or nest. Nest form varies greatly between species in size, shape and substrate. Nests can be very large, for example, males of the Lake Malawi species *Oreochromis* (*Nyasalapia*) *lidole* have been found occupying nests up to 3.1 m in diameter, 1.1 m deep, with a flat central platform of 75 cm across (Turner *et al.* 1991b). Nesting males are often said to crowd together, forming 'leks' or breeding arenas, to display to females (Fryer & Iles 1972). However, nests also occur well apart, and it is not certain that male aggregation is anything other than a reflection of high population density and the lack of alternative suitable pit-digging sites (Turner 1995).

During mate selection, the *Oreochromis* female briefly passes from one territory to another being courted by several successive males. Both male size and territory quality are important in female preference (Turner 1986; Nelson 1995). Pre-fertilisation behaviour is brief and not particularly complex, consisting of a sequence of behaviours (see Chapter 2). Mate recognition is accelerated by a good intraspecific communication system consisting of visual information, behavioural patterns, morphological characteristics and chemical cues (Baerends & Baerends-van Roon 1950; Neil 1964; Falter & Dufayt 1991). Post mating barriers are not well developed in most species, and stable behavioural barriers prevent hybridization in sympatric species.

Once the female is stimulated, a batch of eggs is laid, which is immediately picked up while the male crosses the nest again ejecting sperm. The elaborate genital tassel of the *Oreochromis* (*Nyasalapia*) species may simulate a cluster of eggs, and as with the 'egg dummy' spot on the anal fin of other cichlid species, stimulate the female to collect the eggs as soon as they are laid, providing instant protection and ensuring fertilisation takes place in the mouth. The sequence can be repeated with the same, or a different male (i.e. successive polygyny and polyandry) until all the eggs are laid, often resulting in a clutch where the paternity may be diverse. Molecular markers have been used to confirm multiple paternity within a single brood of maternal mouthbrooding haplochromines (Kellogg *et al.* 1995).

After spawning, the females leave the area of male territories and generally form into small groups. When the young require exogenous food the females move singly into shallow, usually vegetated, areas (Lowe-McConnell 1959). The fry are released and periodically taken back into

the mouth at times of danger (Fryer & Iles 1972; Russock 1986). While guarding fry the female becomes territorial and develops a characteristic fry-guarding pattern of dark stripes across the forehead, dark opercular and chin and a dark eye (Neil 1964). The length of parental care varies between species, lasting from three weeks, as in *O. niloticus*, up to six weeks in *O. (Ny.) squamipinnis* (Trewavas 1983). The high level of parental care allows aquacultural breeders to quickly raise thousands of young for directed selection or for stocking into production units.

1.5.3 Speciation in *Oreochromis*

The great majority of *Oreochromis* species are geographically isolated, and have evolved allopatrically, with one species of a genus or subgenus in any one river-system or groups of systems (Trewavas 1983). The few cases of sympatric speciation include the *Oreochromis (Nyasalapia)* of Lake Malawi (introduced in Section 1.5.5) and of the Malagarazi swamps, Tanzania. The two species of *Oreochromis (Nyasalapia)* that exist together in the Malagarazi swamps (*O. malagarasi* and *O. karomo*) differ strongly in jaw and pharynx dentition and in jaw shape. In Lake Jipe, in the Pangani system, two related species of *Oreochromis* also exist together (Trewavas 1983). Examples of sympatric speciation are also found in *Sarotherodon* and *Tilapia* in the volcanic crater lakes of Cameroon (Trewavas 1983; Schliewen *et al.* 1994).

Allopatrically related species of *Oreochromis*, for example, include *O. mortimeri* that represents *O. mossambicus* in the middle Zambezi, and *O. shiranus* that represents *O. placidus* in the Upper Shiré and Lake Malawi, Chilwa and Chiuta (Trewavas 1983; Sodsuk *et al.* 1995). Geographically isolated populations, which do not exchange genes because of extrinsic barriers, present a problem when applying the biological species concept. The evolutionary significance of such populations comes further into question if genes are exchanged when extrinsic barriers are removed (Butlin & Ritchie 1994). The apparent ease with which species of *Oreochromis* hybridize, and increasing concern for the maintenance of their diversity, brings into question how speciation has occurred and how reproductive isolation is maintained in this group of fish.

Divergence between isolated populations could result from genetic drift, selection for adaptation to environmental differences and sexual selection (Mayr 1963; Schluter 1996). In maternal mouthbrooders, where females visit several males before mating with the preferred one, sexual selection is strong and can probably account for many of the morphological differences between species. The overlapping distribution of some *Oreochromis* species, such as that of *O. mossambicus* and *O. placidus* in the lower Zambezi to Sodwana, suggest that these may have

evolved allopatrically and come into secondary contact (Trewavas 1983; Sodsuk *et al.* 1995). Behavioural reproductive isolation between such species could have evolved in allopatry as a by-product of genetic or ecological divergence (Hinde 1959; Carlstead 1982), or as a consequence of sexual selection (Lande 1980, 1981; West-Eberhard 1983; Lande & Kirkpatrick 1988). Reproductive character displacement may produce divergence in mate recognition systems if interpopulation matings occur when two populations meet after a period of divergence in allopatry (Butlin 1987). At present, allopatric species of *Oreochromis* appear not to have diverged sufficiently in mate recognition systems to prevent interspecific matings.

1.5.4 The aquaculture and introduction of tilapia

Oreochromis species are the most important tilapiine cichlids used in aquaculture; they show excellent growth rates on low protein diets, tolerate wide ranges of environmental conditions (Wohlfarth & Hulata 1981), are amenable to handling and captivity, have a short generation time and breed in captivity (Fryer & Iles 1972; Pullin 1985). However, there are several problems associated with the aquaculture of tilapia. The morphological similarity of commercially important tilapia, has often complicated the culture of tilapia due to the problems associated with species identification. Breeding colouration cannot always be relied on for species identification, particularly in females and immature males. Morphological and meristic characters often have overlapping distributions, and are affected by environmental factors. With the widespread distributions of many species, fish are often reared under different conditions to those which they were originally described. Due to the lack of post-zygotic isolation, the culture of fish of unknown identity and the introduction of tilapia to regions where they are not endemic, has commonly resulted in hybridization (Section 1.5.4.1). The aquaculture of tilapia has been hampered by another physical property of tilapiines; their ability to mature precociously (Section 1.5.4.2). As with the aquaculture of all species, where founder populations have been small, limited broodstock diversity has also had adverse affect on the aquaculture of tilapia. The Nile tilapia (*O. niloticus*) is the main species used in aquaculture and cultured stocks have been propagated around the world. Most of these stocks originated from the same sampling areas in the Nile and Volta rivers and were founded with a limited number of individuals, thereby resulting in a rapid loss of variation within populations through genetic drift. Consequentially, many cultured stocks of tilapia have exhibited poor fitness traits such as growth rate and disease resistance (Pullin & Capili 1990).

1.5.4.1 Hybridization and introductions in tilapia

The culture of fish of unknown identity, and the introduction of tilapia to regions where they are not endemic, have allowed the unintentional hybridization between and among culture and wild fish populations (Daget & Moreau 1981; Macaranas *et al.* 1986). Problems of identification are further complicated by the presence of hybrids which are usually intermediate in appearance to the parental species and even more difficult to identify (McAndrew & Majumdar 1983). The intentional hybridization by fish farmers, in order to produce all male populations, has also made it difficult to determine the genetic purity of populations. Use of molecular markers has greatly improved the ability to identify culture and wild species and hybrids, removing the need to rely solely on morphological and meristic characters. Macaranas *et al.* (1986), using allozyme analysis, found that variability in Philippine *O. niloticus* populations was caused primarily by the introgression of *O. mossambicus,* due to poor broodstock management, resulting in poor or variable growth performance, and widespread interbreeding. Hybridization between culture and wild stocks and also between wild stocks, has been found in many tilapia stocks all over the world (Taniguchi *et al.* 1985; Macaranas *et al.* 1986; De Silva & Ranasinghe 1989). The maintenance of pure species (the 'genetic resource') is very important for fish breeding and stock improvement work, requiring sound knowledge of the genetic status of both wild and cultured populations.

Hybridization between species of tilapia has commonly occurred following introductions and transfers. In Africa, most of the known transfers of tilapia occurred in the 1950s and 1960s, primarily to sustain or increase production (Ogutu-Ohwayo & Hecky 1991). The biology of the introduced species and the ecology of the stocked body of water were often unknown. Hybridization and the similarity in ecological requirements of some tilapiines has led rapidly to competitive exclusion and the elimination of one or more species following an introduction (Trewavas 1983). In Lake Itasy (Madagascar), the introduction of *O. niloticus* caused in a few years the elimination of *O. macrochir* (also introduced). At first, hybrids were frequent, but disappeared later on. Most of the remaining *O. niloticus* have a poor growth rate (Daget & Moreau 1981). It has often been difficult to separate the effects of hybridization from competition following an introduction (Carvalho & Hauser 1995). For example, *Oreochromis spilurus* was an abundant introduced species in Lake Naivasha (Kenya) during the 1950s and 1960s (Siddiqui 1977). In 1956, *O. leucostictus* was introduced and hybridized with *O. spilurus*, resulting in the subsequent disappearance of *O. spilurus*, and later also of the hybrids. Siddiqui (1977) attributed the disappearance of *O. spilurus* to the loss of spawning sited due to the rising level of the lake,

but competition with *O. leucostictus* may also have been involved (Ogutu-Ohwayo & Hecky 1991). In Lakes Victoria and Kyoga, hybridization and competition with introduced *O. niloticus* and *Tilapia zillii* may have contributed to the disappearance of the endemic *O. esculentus* and *O. variabilis* (Welcomme 1966; Ogutu-Ohwayo & Hecky 1991). Hybridization between introduced species may give rise to hybrids which prove to be more of an ecological problem than the parental species. In Australia, *O. mossambicus* has established feral populations and potentially hybridized with at least one other introduced species of *Oreochromis* (*O. hornorum, O. niloticus* and/or *O. aureus*) (Mather & Arthington 1991). After the experience of introduced carp giving rise to tolerant vigorous hybrids, *O. mossambicus* has been declared a noxious species in northern Australia and in New South Wales, with heavy penalties for translocation and cultivation (Arthington 1991).

Apart from the elimination of native species, the introduction of tilapia has resulted in ecological change through the removal, and therefore decrease, of aquatic plants - decreasing the available breeding areas for some native species. There is increased competition for food, space and breeding areas, often changing the biology and breeding behaviour of the native species (Moreau 1983). In Lake Victoria, the loss of genetic diversity (due to a complex combination of factors, including species introductions, overfishing and habitat changes) has been accompanied by a loss of trophic diversity. The transformation of the fish community coincided with profound eutrophication which might be related to alterations of the lake's food-web structure (Ogutu-Ohwayo & Hecky 1991). The transfer of *Oreochromis* species throughout the tropics and subtropics has disrupted the indigenous fauna and flora and destroyed much of the aquaculture and fisheries by out-competing more desirable fish (Pullin 1985; Pullin *et al.* 1997). For example, *O. niloticus* has replaced some native species and is reported as a pest in open waters in Thailand (Welcomme 1988; De longh & Van Zon 1993). *O. mossambicus* has often caused considerable damage to fisheries, for example in some countries the culture of milkfish (*Chanos chanos*) has been disrupted or destroyed (Welcomme 1988; Pullin *et al.* 1997).

1.5.4.2 Precocious maturation and monosex culture

Tilapia were cultured for many years in the limited space of aquaculture ponds, resulting in an enormous quantity of small fish with little economical importance. The transfer of *O. mossambicus* throughout the tropics and subtropics gave tilapia a bad reputation, typified by stunted, black, unattractive fish, that bred prolifically (Pullin 1985). Precocious maturation, an ecological and environmental advantage, allows tilapia to mature and breed in small ponds and

lagoons which might dry out. However, in aquaculture, precocious maturation is an undesirable feature since breeding at a small size diverts energy from growth into reproduction (territorial, courtship behaviour and the metabolic cost of gamete production). Furthermore, the progeny produced by the stocked fish compete for available space and food resources, thereby inhibiting the growth of the stocked fish, especially in ponds, where space and food quickly become limited.

The culture of tilapia made significant progress when it was shown that hybridization between some species of this genus gave rise to fertile F_1 hybrids, which present unusual sex ratios, including 100% males (Lovshin & De Silva 1975; Wohlfarth & Hulata 1981; Avtalion 1982; Wohlfarth 1994). The monosex culture of tilapia prevents their uncontrolled reproduction. Several artificial crosses of tilapia species produce only female progeny, but because male tilapia also grow faster than females, most interest has centred around crosses in which there is a predominant or complete maleness in the F_1 generation (Elder *et al.* 1971). In addition to facilitating monosex culture, such crosses are of value since the progeny can exhibit up to twice the growth rate of the parental stock due to hybrid vigour. However, the widespread adoption of interspecific hybrid production can result in the introgression of tilapia species, with deleterious implications for the conservation of tilapia genetic resources (Mair & Little 1991). The consistent production of all-male broods by interspecific hybridization depends on the genetic purity of the parental stocks. However, the production of 100% males is rare (especially if parental stocks are not pure) and the accidental introduction of even one female tilapia (it is difficult to sex immature fish) can result in the original problem (Wohlfarth 1994).

Other, non-genetic, methods which have been used for population control include manual sexing, cage culture, the use of predators, and high density stocking - all of which have proven to be ineffective or inefficient (Wohlfarth & Hulata 1981; Mair & Little 1991). Most advances in the aquaculture of tilapia have been through genetic manipulations (including interspecific hybridization). These have involved chromosome manipulation (polyploidy and gynogenesis) to produce sterile progeny (Mair 1993), hormonal sex reversal (Mair & Little 1991), and the generation of all-male producing broodstocks through 'YY, supermale' production (Mair *et al.* 1992, 1997). The large-scale production of genetically male tilapia, through the YY supermale technology, has allowed the full potential of tilapia aquaculture to be realised. However, since such technology is greatly dependent on the financial and scientific resources available, precocious maturation remains to be a problem for the poor small-hold farmer (Little 1998).

1.5.5 The aquaculture and fisheries of tilapia in Malawi

Lake Malawi, the ninth largest lake in the world (length 600 km, maximum width 80 km, maximum depth 700 m), covers an area of about 31,000 km^2 and is bordered by Malawi, Tanzania and Mozambique (Konings 1990) (Figure 1.1, and Figure 3.4, Chapter 3). Estimates for the age of Lake Malawi vary, ranging from 1-2 × 10^6 years old (Fryer & Iles 1972) to 4.5 - 8.5 × 10^6 years old (Martens 1997), and the age of the species flocks is also controversial. Based on evidence for low-lake strands during historic times (Owen *et al.* 1990), the formation of dozens of (sub-) species of cichlids in the southern part of the lake possibly occurred in only a few centuries (Meyer 1993; Parker & Kornfield 1997). There are reported to be over 600 species of haplochromine cichlids in Lake Malawi (Konings 1989; Turner 1997), whereas only five tilapiine species have been described. However, tilapia are of major economic importance to the area surrounding Lake Malawi, both in fisheries (although presently declining) and increasingly in aquaculture (Kaunda 1994; Brummett 1995; Maluwa *et al.* 1995).

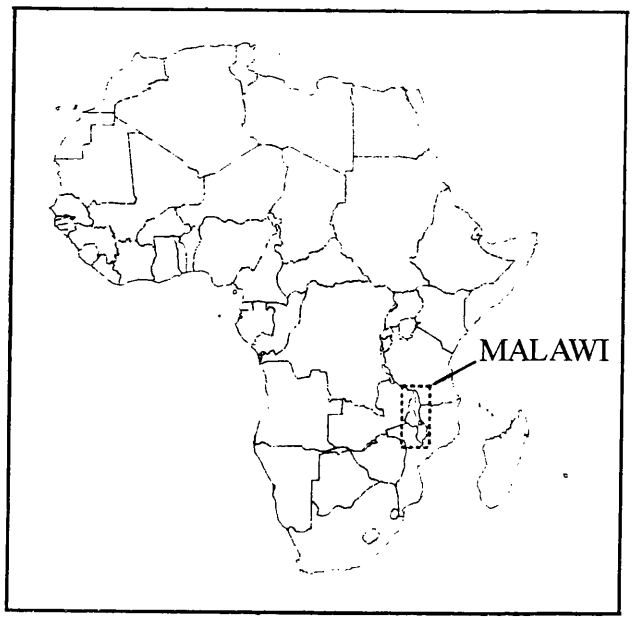


Figure 1.1 Geographical location of Malawi

The tilapiine cichlids found in Lake Malawi and its surrounding lakes and rivers include *Tilapia rendalli* (Boulenger), which is widely distributed throughout Africa, and *Oreochromis (Oreochromis) shiranus* (Boulenger) which is endemic to Lakes Malawi, Chilwa and Chiuta and the Shiré River (Sodsuk *et al.* 1995). Trewavas (1983) placed *O. shiranus* in the subgenus *Oreochromis* VII. Two subspecies are recognized, *O. shiranus shiranus* (Boulenger) and *O. shiranus chilwa* (Trewavas), separated mainly by male breeding colours (Trewavas 1983). The other tilapiine species comprise a monophyletic flock, of the subgenus *Nyasalapia,* which exhibit many unique synapomorphic characters when compared with the other East and Central *Oreochromis (Nyasalapia)* species (Trewavas 1983). Lake Malawi is the only large species-rich lake to contain a tilapia flock, collectively known as 'chambo', which may have evolved within the lake in the presence of competing haplochromines. Trewavas (1983) described four different

species; *Oreochromis (Nyasalapia) karongae* (Trewavas), *O. (Ny.) lidole* (Trewavas), *O. (Ny.) saka* (Lowe) and *O.(Ny.) squamipinnis* (Günther). In a study of morphology and taxonomy, Turner & Robinson (1991) found considerable habitat-related geographic variation in *O. karongae*, and concluded that *O. saka* was the southern form of *O. karongae* rather than a separate species. This classification of three, rather than four, chambo species has been adopted for this study.

The subgenus *Nyasalapia,* most recently classified by Trewavas (1983), includes all *Oreochromis* species which have males with a tasselled genital papilla. Thys van den Audenaerde (1968) restricted the subgenus *Nyasalapia,* as part of the genus *Tilapia,* to the Lake Malawi species only. He proposed that all other tasselled male species form the separate subgenus *Loruwiala*, of which *O. macrochir* is the type species (found in river systems to the East, West and North of Lake Malawi). Sodsuk *et al.* (1995), in a comparison of allozyme data from the Lake Malawi *Oreochromis (Nyasalapia)* with five species from the same sub genera, support the hypotheses of both Trewavas (1983) and Thys van den Audenaerde (1968) that chambo form a monophyletic group, and that *O. macrochir* or a related species represents the sister taxon.

The breeding and feeding habitats of all *Oreochromis* species in Lake Malawi overlap, although the distribution of *O. shiranus* is more restricted than that of chambo. *O. shiranus* generally remains in shallow vegetated areas throughout its life, breeding inshore (to 4 m deep) in sand or mud-banks near reeds (Trewavas 1983). *O. shiranus* is believed to have entered Lake Malawi from the south and become sympatric with the *Oreochromis (Nyasalapia)* only when Lake Malawi expanded southwards in the Pleistocene (Trewavas 1983). A great overlap is observed between breeding sites, breeding season and morphology of chambo species. All chambo species breed in a variety of substrata, including mud, clean sand and rocky beaches (Turner *et al.* 1991b). *O. karongae* is the most inshore living of chambo species, breeding in water between 0.5 to at least 28 m (Turner *et al.* 1991b). *O. squamipinnis* also breeds in water less than 4 m deep (Turner *et al.* 1991b), but has been found breeding between 16 and 20 m (Lowe-McConnell 1959). *O. lidole* is the most offshore living species, breeding at depths no shallower than 17 m, up to perhaps 50 m (Turner *et al.* 1991b). It is uncertain what factors maintain reproductive isolation between chambo species or how they evolved (Turner *et al.* 1991a, b), although it is unlikely that they are, or have been geographically isolated, which suggest that these species may have evolved in sympatry (discussed in Section 6.3, Chapter 6).

In Malawi, fish provides an estimated 70% of the available animal protein to the nation, of which the endemic tilapia support a fishery of 10,000 tonnes per annum (1980s) and have a cash

value three times higher than other fish species (Kaunda 1994). Despite such an enormous contribution, the fisheries department has always been marginalized in terms of financial resources. Chambo, which form the basis of a traditional dish, are a presently declining component of the fishery in Lake Malawi due to overfishing and the destruction of nests by beach seining (Turner 1995). As the population of Malawi grows (estimated to be over 11 million by the year 2000) the supply of fish protein is likely to become increasingly reliant on aquaculture (Brummett 1995).

Aquaculture forms only a small activity of the Fisheries Department and has similarly suffered from limited funding. Due to a number of externally funded projects (GTZ/ICLARM, EEC and German government) aquaculture is expanding rapidly, but production is still relatively negligible. Total fish production in the small-scale sub-sector rose from 40 t in 1988 to about 140 t in 1993 (Kaunda 1994). More recently, the Malawi Government has developed an aquacultural policy to encourage, where appropriate, fish farming as a means to supplement fish production from capture fisheries. Despite an increase of fish farmers to over 2000 in 1995, fish production from aquaculture has still been relatively low (Maluwa *et al.* 1995). There has been most return, at smallholder level, when fish farming is integrated with other agricultural activities, such as arable crop and livestock farming, whose waste products are important farm inputs to aquaculture (Brummett 1995). *O. shiranus* has been distributed widely as a farmed species throughout Malawi and often stocked into small water bodies (Mattson & Kaunda 1997). *O. karongae, T. rendalli* and *O. shiranus,* are commonly grown in monoculture, or in polyculture using the catfish *Clarias gariepinus* with either *O. shiranus* or *O. karongae* (Maluwa *et al.* 1995). Many fish farms, particularly experimental ones, keep both *O. shiranus* and chambo species (Maluwa & Dickson 1993; Mattson & Kaunda 1997). There has been anecdotal evidence (based on morphological characters) that the introduction of *O. karongae* into ponds holding *O. shiranus* has resulted in the formation of interspecific hybrids (A. Brooks & G. F. Turner; *pers. comm.*).

1.6 PROJECT AIMS

The specific aims of this project are:

1) *To investigate mate choice in* Oreochromis *species and their hybrids* - There have been no comprehensive studies of mate preference in pure-bred and hybrid females of *Oreochromis,* when given a choice of pure-bred and hybrid males, and of the behavioural interactions between courting fish. Such a study would be valuable in determining the effects of hybridization on the population dynamics of pure species, and in predicting the fate of the hybrid population. If fertile hybrids

prefer hybrids as mates, and discriminate against parental species, this may reduce introgression and allow the coexistence of parental species and hybrids. Analyses of behavioural interactions between males and females of *Oreochromis* species and their hybrids may also give further insight into the role of ethological barriers to hybridization and determine if isolating barriers between allopatric species are purely geographic.

2) *To investigate the incidence of hybridization between endemic species of* Oreochromis - The presence of hybrids between *Oreochromis* species in farm ponds is of concern to their aquacultural performance and, in the event of hybrids escaping, to the unique endemic species of Lake Malawi. Three methods will be employed to investigate the incidence of hybridization between *Oreochromis (Nyasalapia)* and *Oreochromis (O.) shiranus*: allozyme electrophoresis, RAPD analysis and morphological analysis. Both methods of molecular genetic analysis are relatively easy and quick procedures. Unlike molecular techniques, morphological analysis is an inexpensive method, requiring relatively little expertise and can be used in the field. The following aims apply:

i) To establish whether hybrids are fertile, based on the presence of post-F_1 generation hybrid individuals.

ii) To compare RAPD, allozyme and morphological data on the incidence of hybridization, and to evaluate the efficiency of each technique in identifying hybrids.

iii) To investigate the genetic status of the *Oreochromis* species in wild and farm pond populations, and to examine the level of genetic differentiation between *Oreochromis* species and wild and farm pond populations.

iv) To examine the effects of stocking history (e.g. species used, density, sex ratios) and environmental conditions (e.g. water clarity, nesting area available *etc.*) on the incidence of hybridization in farm ponds.

v) To identify possible avenues for the escape of culture fish into the wild, and assess the evolutionary implications of any such escape on the native *Oreochromis*, and on the other endemic species of Lake Malawi.

vi) To evaluate the process (in particular the role of courtship behaviour and sexual selection) by which reproductive isolation may have arisen and is maintained between species of *Oreochromis* in Lake Malawi.

CHAPTER 2
MATE CHOICE IN PURE-BRED AND HYBRID FISH OF TWO *OREOCHROMIS* SPECIES

2.1 INTRODUCTION

In maternal mouthbrooding tilapiines (*Oreochromis* species), the males establish and defend courtship territories. The territories, often in dense aggregations, are sites only for mating and are not used for rearing offspring (Baerends & Baerends-van Roon 1950; Fryer & Iles 1972). The spawning pit is the central focus of a male's territory, from which it displays to approaching females. The close proximity of many males in the spawning 'arena' provides females ample opportunity to choose among them. Both female choice, and male-male competition in the establishment and maintenance of territories, are important components of sexual selection (Turner 1986; Nelson 1995). Sexually selected characters, such as colour patterns, spawning pit characteristics and secondary sexual structures (e.g. genital papillae) often also play a dominant role in mate recognition. Other cues which contribute to the communication system of these species include chemical (Falter & Dufayt 1991) and possibly auditory cues (Baylis 1976a, b). As with other cichlids, postmating barriers are not complete in *Oreochromis* species. Behavioural patterns, and other forms of visual information, are potential isolating mechanisms which operate early in courtship, thereby reducing the risk of wasting energy with a mate of the wrong species and gamete wastage due to inviability of hybrid offspring.

Isolating mechanisms operative amongst sympatric species (whether ethological, ecological or temporal), commonly do not exist amongst formerly allopatric species. This is particularly true of *Oreochromis*, where hybridization between previously allopatric species has commonly occurred following introductions (Moreau 1983; Ogutu-Ohwayo & Hecky 1991), in aquaculture (Elder *et al.* 1971; Macaranas 1986) and in the stocking of reservoirs (DeSilva & Ranasinghe 1989; Gregg *et al.* 1998). Hybridization also occurs amongst sympatric species of *Oreochromis* when they are unnaturally confined in aquacultural ponds with high density (present study: Chapters 3 - 5). Behavioural studies have shown that *Oreochromis* species have the ability to discriminate between conspecific and non-conspecific mates by means of visual cues alone, although occasional 'errors' in the choice of mate are made (Falter & Charlier 1989). Generally few differences have been found in the form of behaviours between formerly allopatric species (Elder *et al.* 1971; Falter & Charlier 1989). Species-specific differences in the threshold of aggressive and sexual behaviour,

rather than differences in the form of behaviours, have been used to explain incompatible heterospecific encounters between *Oreochromis* species (Baerends & Blokzijl 1963; Falter & Charlier 1989; Falter & Dufayt 1991) and other species of cichlid (Baylis 1976b). Falter & Dufayt (1991) suggested that species-specific differences in colour patterns only play a secondary role to differences in aggression.

Despite the common occurrence of hybridization between *Oreochromis* species and the subsequent production of frequently viable hybrid offspring, few studies have investigated the choice of mate between pure species and hybrids. Falter & Charlier (1989) found that hybrid females spawned significantly more frequently than pure species, but failed to show any preference between males of the two parental species (*O. niloticus* and *O. mossambicus*). The mating preference of hybrids towards their own males could not be analysed because hybrid males had grown much faster than pure males. A tendency to prefer hybrid males to pure-bred males has been reported in haplochromines (Crapon de Caprona 1986b). However, there appear to have been no comprehensive investigations into mate choice between pure and hybrid fish of *Oreochromis*. Such a study would be valuable in determining the effects of hybridization on the stability of the populations of pure species, and in predicting the fate of the hybrid population in situations where two species come into contact through introductions or aquaculture. If fertile hybrids prefer to spawn with each other (i.e. assortative mating), and discriminate against parental species under free choice conditions, this may reduce introgression and allow the coexistence of species and hybrids (Crapon de Caprona 1986a, b). Analyses of mate choice in *Oreochromis* species and their hybrids may also give further insight into the role of ethological barriers to hybridization.

The courtship behaviour of *Oreochromis* species has been most closely studied in *Oreochromis mossambicus* (Baerends & Baerends-van Roon 1950; Neil 1964; Turner 1986). In this species, courtship is reduced to a minimum in an almost ritualised sequence of behaviours, and preliminary displays may be omitted if the visiting female is showing the appropriate colour pattern and behaviour. If the female is not receptive, the male may repeat a previous step in the sequence or chase the intruder away if aggressive displays are returned. As the female approaches the male's territory, the male swims out of its spawning pit towards the female and adopts a head-down posture. The courting male performs a quick tail-quivering display, and attempts to 'lead' the female to the spawning pit. The male breaks away from the female to swim toward the pit with exaggerated swimming movements, and when returning to the female generally performs a tail-beating display. This sequence of displays may be repeated until the female follows the male into

the spawning pit, or is chased away if unreceptive. At the spawning pit, the male initially circles around in a head-down posture and then performs a skimming motion with his genital papilla over the substrate. Receptive females generally circle with the male and perform skimming movements and mouth-digging. These activities continue until spawning occurs. The female lays a batch of eggs and immediately begins to pick them up in her mouth, while the male is fertilising them. Pair-bonds are absent; the male may spawn with several females in quick succession, and the female may lay batches of eggs with different males during a single spawning.

In the present study, mate choice and courtship behaviour (agonistic and courtship acts) were investigated in hybrids, and pure species of *Oreochromis mossambicus* and the closely related but more highly coloured, *Oreochromis spilurus*. As with the majority of *Oreochromis* species which occur together in aquaculture or are introduced into the range of a native species, these species naturally occur allopatrically. *O. spilurus* is endemic to the lower reaches of rivers from Kenya to Tanzania, and *O. mossambicus* naturally occurs from the Lower Zambezi up to Tete and the coastal parts of Mozambique. Of the species inhabiting the lower parts of the eastward-flowing rivers of east Africa, forming the '*mossambicus* complex', *O. spilurus* is the northernmost species and *O. mossambicus* is the southernmost (Trewavas 1983). Fish of this complex are thought to have moved along the eastern coast of Africa from estuary to estuary especially during times of flood (McAndrew & Majumdar 1981). *O. spilurus* and *O. mossambicus* are not generally farmed together, although *O. mossambicus* has frequently hybridized with *O. niloticus* in aquaculture and hybrid progeny have escaped to the wild (Taniguchi *et al.* 1985; DeSilva & Ranasinghe 1989). *O. spilurus* is not commonly used in aquaculture, although the widespread stocking of *O. spilurus* subspecies in Kenya has made it difficult to find there any waters where a pure population of *O. s. niger* exists without admixture with *O. s. spilurus* or other species (Trewavas 1983).

The aims of this study were:

1) To determine if pure-bred species (*O. spilurus* and *O. mossambicus*) mate assortatively and behave differently (in the form and frequency of agonistic and courtship behaviours) to conspecifics and pure-bred heterospecifics.

2) To determine if pure-bred species avoid mating with hybrids, and behave differently to hybrids and conspecifics.

3) To identify the mate preference of hybrid females - whether they prefer hybrids or the parental species, and if they prefer one of the parental species over the other.

4) To determine if assays of behaviour can predict the final result of the spawning decision in pure-bred species and hybrids.

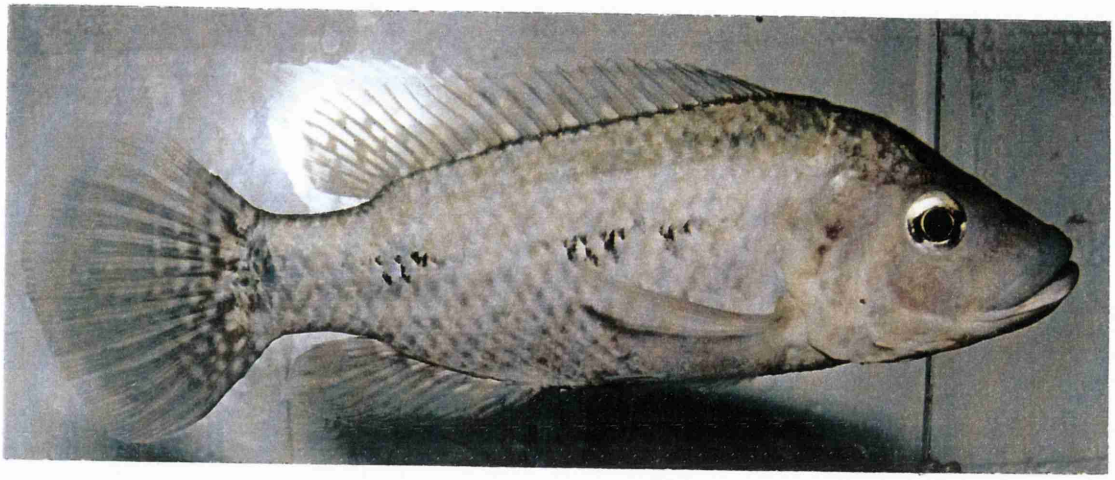
5) To assess the consequences of mate choice between pure species and hybrids on populations where hybridization has occurred through aquaculture or introductions, and thereby determine the fate of hybrid populations.

2.2 MATERIALS AND METHODS

2.2.1 Experimental fish

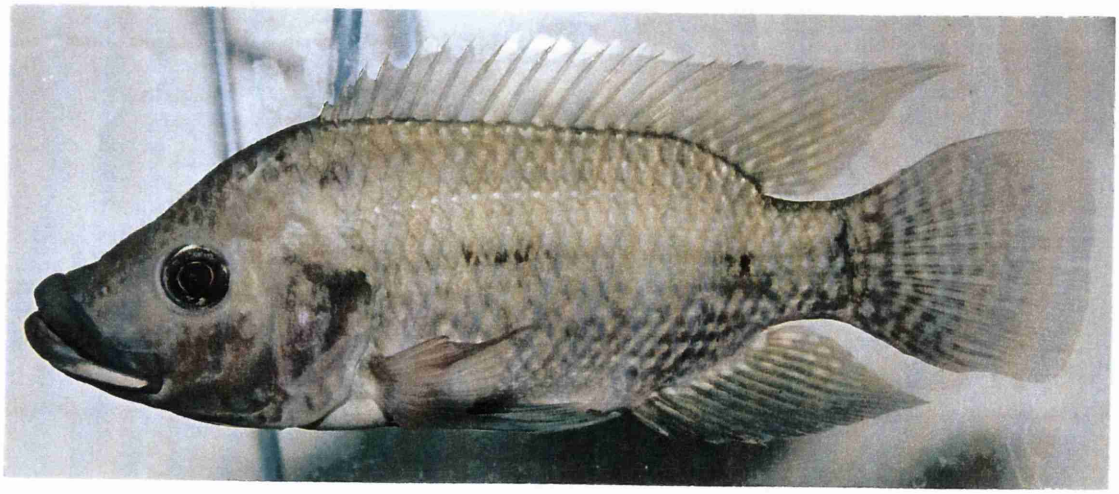
The fish used in the following experiments were first and second generation laboratory stock from the Tilapia research laboratories at the Department of Biological Sciences, University of Wales Swansea. Stocks of *Oreochromis spilurus* originated from wild fish collected in Kenya, and *Oreochromis mossambicus* were from an aquacultural strain (both presumably pure-bred stocks). First generation hybrids, between an *O. spilurus* female and *O. mossambicus* male, were produced through artificial fertilization. Fish were raised in mixed sex groups, but the three types of fish (two pure species and hybrids) were kept in separate 50-gallon plastic tubs connected to a common water circulation system. Prior to the mate choice experiments, sexually mature (virgin) fish were randomly chosen from the stock tubs and introduced to the aquarium to allow them to habituate and assume breeding colouration.

The breeding *O. mossambicus* male is black with white lower parts to the head, and red margins to the dorsal and caudal fins (Baerends & Baerends van Roon 1950; Neil 1964). The intensely black colouration was rapidly lost when the *O. mossambicus* male became frightened, making it difficult to provide a photograph of a male in full breeding colouration (Figure 2.1a). The breeding colouration of the *O. mossambicus* male contrasts highly to those of the *O. spilurus* male, which has yellow green lower flanks and a pink orange operculum (Figure 2.1c). The dorsal and caudal fins of the breeding *O. spilurus* male have red or orange margins, and bright sky-blue areas or spots are found on the dorsal, anal and pelvic fins (Trewavas 1983). The colouration of breeding hybrid males (Figure 2.1b) was largely intermediate between that of the parental species, possessing the more colourful fin colouration of *O. spilurus,* but the overall darker appearance of *O. mossambicus*. The concave snout shape and thick lips of the hybrid males was more similar to the features of *O. spilurus* males than those of *O. mossambicus*. The *O. mossambicus* female (Figure 2.2a) is silvery with mid-lateral blotches, and becomes more silvery as reproductive condition is reached (Baerends & Baerends van Roon 1950). The sexually mature *O. spilurus* female is yellow green (Figure 2.2c). The hybrid female (Figure 2.2b) had the yellow green appearance of the *O. spilurus* female, with the silvery sheen of the *O. mossambicus* female.



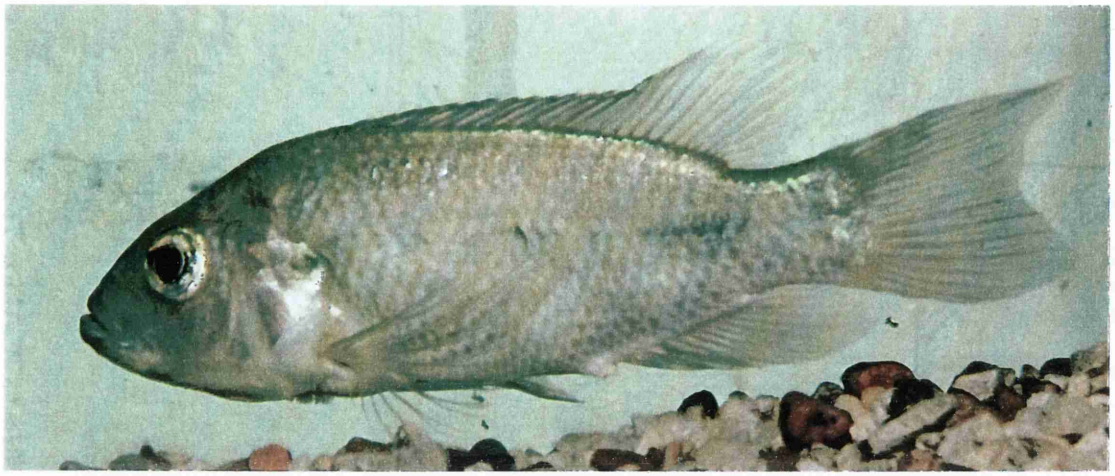
a)

b)

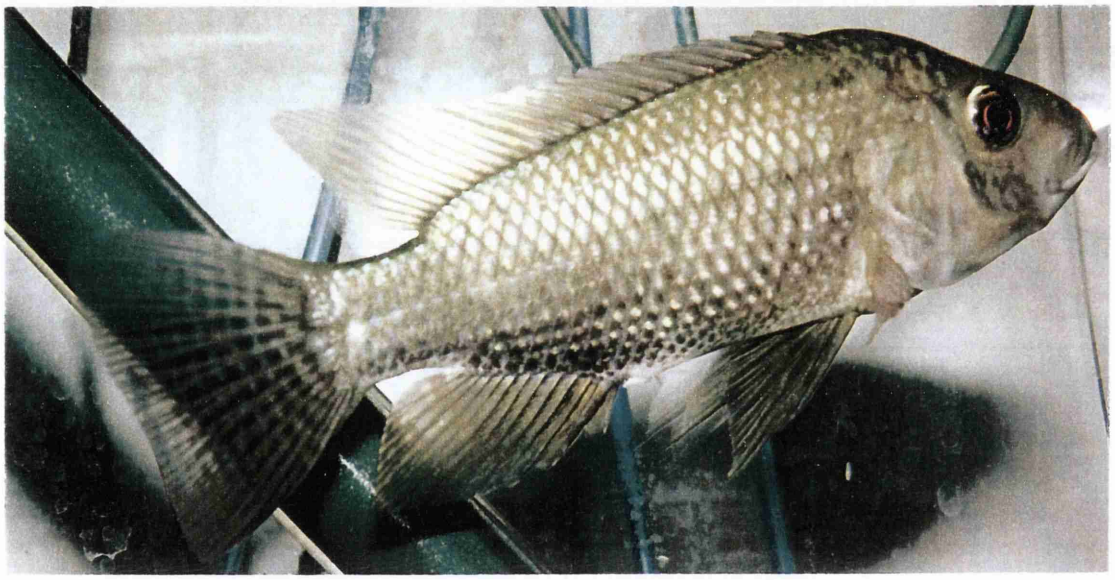


c)

Figure 2.1 *Oreochromis mossambicus* male (a), hybrid male (*O. spilurus* female x *O. mossambicus* male) (b), *Oreochromis spilurus* male (c) (Photographs by David Tavner).



a)



b)

c)

Figure 2.2 *Oreochromis mossambicus* female (a), hybrid female (*O. spilurus* female x *O. mossambicus* male) (b), *Oreochromis spilurus* female (c) (Photographs by David Tavner).

2.2.2 Experimental Design

All mate choice experiments were carried out in aquarium tanks measuring 122 cm (length) x 30 cm (width) x 38 cm (height), each equipped with one water heater and two airlift tubes (at each rear corner of the tank, providing approximately equal water movement) attached to an under-gravel filter plate (Figure 2.3). The filter plate was covered with a 5 cm deep layer of course-grain gravel. Water temperature was maintained at approximately 28°C, and a 14 h light : 10 h dark regime maintained. Fish were fed twice daily (morning and evening) with 'Ewos' trout pellets (48% protein); all fish were given equal amounts of food. The tanks were cleaned and the water partially changed between experiments. Each tank was divided into three compartments by two transparent plastic partitions (which were not water-tight), creating one large middle compartment and two smaller end compartments. The partitions were necessary to prevent any fighting between the fish in the enclosed space of captivity. A male was located at either end of the tank, and a removable opaque partition was placed over the transparent partitions. To control for end preference, the type of male (*O. mossambicus*, *O. spilurus* or hybrid) at either side of the tank was alternated. The middle compartment, which contained the female, was further divided by two incomplete opaque plastic partitions which allowed her passage but prevented the males from seeing and, thereby, influencing each other (design adapted from Balshine-Earn & McAndrew 1995).

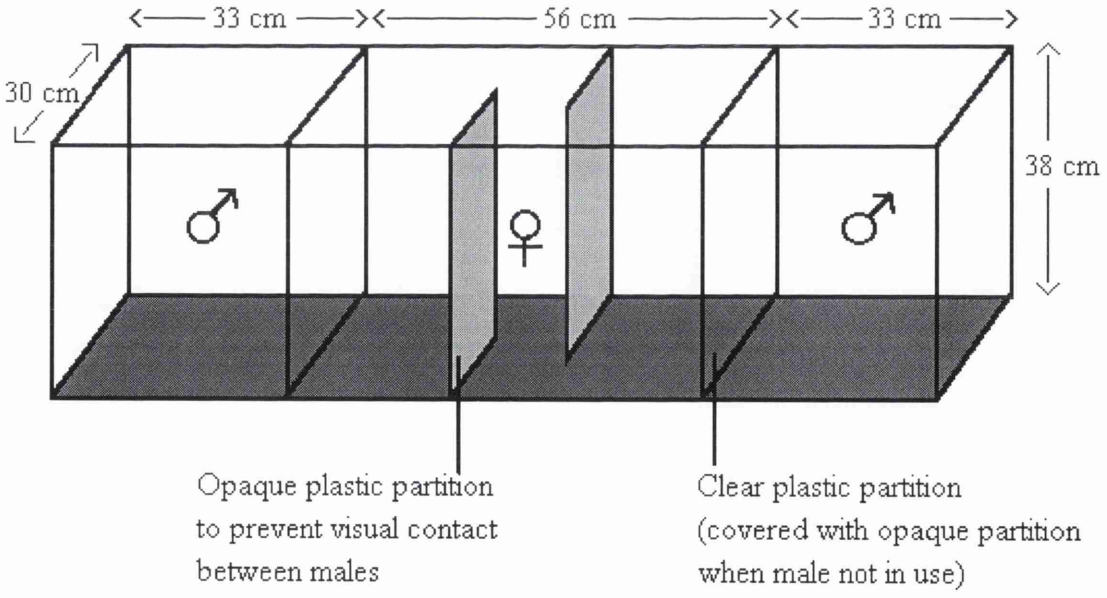


Figure 2.3 Schematic diagram of the mate choice tank set up.

In a total of 54 experiments; nine male and female *O. spilurus*, seven male and female *O. mossambicus*, and six male and nine female hybrid fish were used. A lack of sexually mature fish,

of a similar size, meant that some fish had to be used in more than one experiment. Males were larger than females, but fish of each sex were approximately of the same size (males 17 -19 cm, females 13-16.5 cm). The three types of female were each given a choice of two males from the six possible combinations of the same or different types of *O. spilurus*, *O. mossambicus* and hybrid males. This choice was repeated twice more with different males and females (i.e. three replicates of each combination). Sexually mature fish (two males and one female) were given 24 hours to habituate to the test aquarium. All tests females were ready to spawn and approximately at the same level of ripeness, as based on their colouration and protrusion of ovipositor. Experiments were carried out in the afternoon, since diurnal fluctuations have been found to occur in the territorial behaviour of males and in the spawning activities of the females (Munro & Singh 1987). In order to keep all test conditions constant, spawning pits constructed by males were removed by flattening the gravel. However, this disturbance often upset habituated males (particularly *O. mossambicus* males) which would no longer react to introduced females. Consequently, spawning pits were left undisturbed and their possible influence in mate choice noted.

The experiment was split into two observation periods:

1) **'Forced-choice' experiment** - The female had visual access to only one male for a 15 minute period. The other male was hidden by an opaque partition. All fish had water contact with each other. This was then repeated with the other male. The two types of male were presented in a random order. All three fish had to be introduced into the tank at the same time, to avoid disturbing them excessively and to give them an equal amount of time to habituate.

2) **'Open-choice' experiment** - The female had visual access to both males for a 30 minute period. As before, the plastic partitions did not prevent water contact between fish. The experiment began once the female had swum into both of the end compartments and viewed each male once.

During the two experiments, the frequency of eight behaviours (aggressive and sexual behaviours) were recorded in both sexes (Table 2.1). The time that the female spent in front of the male, in visual contact, was recorded against the time she spent away from the male. In the Open-choice experiments the time that females spent in visual contact with each male was recorded. Once this experiment was complete the fish were left for a maximum of 10 days, or until the female had chosen a male to spawn with. This was indicated by the construction of a spawning pit near the partition of the particular male chosen, and the presence of eggs in the females mouth. If the female showed no intention of spawning within ten days the experiment was discontinued.

Additional mate choice experiments were carried out, where the three types of female were

Table 2.1 Ethogram of aggressive and sexual behaviours recorded during mate choice experiments, and equivalent behaviours described by other authors.

Behaviour	Abbr.	Description
Biting*	Bi.	Bites with open mouth the partition near the other fish (Falter & Charlier 1989).
Digging†	Di.	Gravel picked up in the mouth, moved or spat out. Performed by males when establishing a territory and building a spawning pit, and by both sexes in courtship.
Frontal display*	F.D.	Directly facing opposite individual with all fins erect, mouth usually open. Described by Neil (1964) during intraterritorial fights between fish of the same sex only. Also observed in females displaying to males (Falter & Charlier 1989).
Lateral display*	L.D.	Flank orientated towards other fish with dorsal, ventral and anal fins erect. May be performed in different degrees of intensity, with fins fully erect and branchiostegal membrane distended at highest degree. Described by Neil (1964) during intraterritorial fights between fish of the same sex only. Also observed in females displaying to males (Falter & Charlier 1989).
Nodding	No.	Head-down posture, with closed unpaired and pelvic fins. Similar to 'tilting' as described by Neil (1964), 'inviting' as described by Baerends & Baerends van Roon (1950) and 'rapprochement' as described by Falter & Charlier (1989). Only the latter authors described this behaviour in females also.
Quivering	Qu.	Like shaking, but more intense smaller oscillations. Similar to 'twitching' as described by Neil (1964) and 'jerking' as described by Baerends & Baerends van Roon (1950).
Shaking	Sh.	Head bent sidewards and brought back alternately to each side, movement may travel down the body. Similar to 'tail-wagging' as described by Neil (1964) and by Baerends & Baerends van Roon (1950).
Skimming	Sk.	Preparatory activity of laying eggs and fertilisation, consisting of a swift movement as the abdomen makes contact with the substrate (Baerends & Baerends van Roon 1950).

Abbr., abbreviation used. * Aggressive behaviours. Sexual behaviours are unmarked. † Digging can be interpreted as an aggressive behaviour in the establishment of territories, or as a sexual behaviour during courtship.

given a choice of two different male types only. For these experiments no behavioural displays were observed; only the male chosen to spawn with was recorded. Experiments were continued until the female spawned (i.e. no time limit). Eight replicates of each of three combinations of males (*O. mossambicus* and *O. spilurus*, *O. mossambicus* and hybrid, *O. spilurus* and hybrid) were conducted, for all three types of female (72 experiments in total). Additional experiments were carried out, where mate choice was based on visual information alone (i.e. no water contact, and

therefore no olfactory contact). Experiments took place in three isolated tanks, where the two tanks containing males were adjoining (although an opaque partition prevented visual contact) and the tank containing the female was placed in front of, and in the middle of, the two other tanks. A total of seven experiments was conducted, where the two pure-bred females were given the choice between a conspecific and heterospecific male.

2.2.3 Data Analyses

2.2.3.1 Mate choice

Chi-squared tests were performed to test for difference in the frequency of spawning between the three types of female in the initial 54 mate choice tests (limited time period of ten days), and to test if the choice of mate was random in the additional 72 experiments (unlimited time period). The number of days taken to spawn by each female type in the additional mate choice experiments were compared using a Kruskal-Wallis test. Mate choice results from initial and additional experiments were combined, and two-way contingency tests between female types, in their choice of spawning partner between male types, were compared using G-tests of independence with the Williams correction (Sokal & Rohlf 1995). Eight tests were performed to test the following null hypotheses.

1) Pure-bred types do not mate assortatively.
2) Pure-bred females do not differ in their preference for conspecific and hybrid males.
3) Pure-bred females do not differ in their preference for heterospecific and hybrid males.
4) *O. spilurus* and hybrid females do not differ in their preference for *O. spilurus* and hybrid males.
5) *O. mossambicus* and hybrid females do not differ in their preference for *O. mossambicus* and hybrid males.
6) *O. spilurus* and hybrid females do not differ in their preference for each type of pure-bred male.
7) *O. mossambicus* and hybrid females do not differ in their preference for each pure-bred male.
8) Hybrid females do not prefer different pure-bred male types over hybrid males.

2.2.3.2 Time allocation

Forced-choice tests

To test for differences in the time that females spent in front of males or away from males, paired comparisons were made using the Wilcoxon signed rank test. Similarly, paired comparisons were made on the time spent in front of the first male and the second male presented to the female.

To investigate whether the amount of time spent in front of a male was affected by the type of female and type of male being tested, the data were subjected to a two-way ANOVA. In order to fulfill the assumptions of this test, the data were transformed. Several methods of transformation were tested to produce equal variances between groups and a normal distribution of data; these parameters were achieved when the original data were cubed. Differences in the time data from experiments where the female was consecutively presented with two males of the same type, to those where the two males were of a different type, were compared using the Mann-Whitney test.

Open-choice tests

During the Open-choice experiments, females were given the choice of two males and the time spent in front of each male during the 30 minute observation period was recorded. All data were subjected to paired t-tests, where the time that the female spent in front of each of the two males was compared. Two-way contingency tests between female types, in their 'choice' between male types (i.e. male with which the longest time was spent) were compared using G-tests of independence with Williams's correction (Sokal & Rohlf 1995). Eight tests were performed to test the null hypotheses addressed in Section 2.3.3.1.

To fully utilise all the data generated from each Open-choice test, they were divided into two sets of data. For instance, if a hybrid female was given the choice of an *O. mossambicus* male and an *O. spilurus* male (located at either end of the tank), the data were considered as:

1) the time that a hybrid female spent in front of an *O. mossambicus* male, when the other male present (or 'O.m.p') was *O. spilurus*; and
2) the time that a hybrid female spent in front of an *O. spilurus* male, when the O.m.p was *O. mossambicus*.

When the female was given a choice of the same two types of male, only one set of data was analysed (chosen randomly). Thereby, the three types of female were presented with nine different combinations of male and O.m.p types. Three replicates of each combination (female, male and O.m.p) produced 81 sets of data. This procedure was adopted for all data analysed from the Open-choice experiments, including the number of behavioural displays. Time allocation data were subjected to a three-way ANOVA, where the type of female, type of male and type of 'other male present' were considered.

The two sets of data generated from each experiment cannot be considered as completely independent; this may affect the statistical robustness of the results. Therefore, three-way ANOVAs were also conducted on data from two separate experiments (both with the same

combination of three fish, but with data used from either male). Unfortunately, with this approach, only one set of data for each experiment was available for analyses, therefore the mean-square of the three-way interaction was used as the error, to calculate *F*-ratios. In all analyses of time and behavioural data, there were very few significant results. The significant results did not contradict the findings of the ANOVAs based on non-independent data, and are therefore not presented.

2.2.3.3 Behavioural displays

Forced-choice tests

Data for each of six behaviours recorded during the Forced-choice experiments were analysed using two-way ANOVAs, where differences were tested in the number of displays performed by the three types of female and 'received' by each type of male, and where interactions between female type and male type were tested for. All behavioural data were tested further using a two-way multivariate analysis of variance (MANOVA). The same analyses were performed on behavioural displays recorded for males. All data had to be transformed (in various ways) to fulfill the assumptions of the test, except for the number of frontal displays performed by females where the original data were used. The Mann-Whitney test was used to compare data (untransformed) from experiments where the female was consecutively presented with two males of the same type, to experiments where the two males were of a different type.

Open-choice tests

Data for each of six behaviours recorded, for males and females, during the Open-choice experiments were tested by three-way ANOVAs, where the type of female, type of male and type of other male present were considered. All behavioural data were tested further using a three-way multivariate analysis of variance (MANOVA). Some data had to be transformed to fulfill the assumptions of the test. The original data were used for the number of frontal displays, lateral displays, nods and shakes performed by females, and the number of nods performed by males. There were insufficient data (i.e. insufficient degrees of freedom) to perform one-way MANOVAs to determine, for example, if the behaviour of the female differs towards the two males presented. Therefore, all behaviours for each test where the female was presented with two different male types were compared using a R X C test of independence using the G-test, with Williams's correction (Sokal & Rohlf 1995). This test was performed on the number of displays, for six behaviours, performed by the female to the two males, and on the number of displays performed by each male to the female.

2.3 RESULTS

2.3.1 Mate choice and frequency of spawning

2.3.1.1 With water and visual contact between fish

Of the 54 initial mate choice experiments, 27 resulted in the female spawning (Table 2.2). The total number of experiments where spawning did and did not occur, within ten days, varied between the three types of female ($X^2 = 16.4$, $P < 0.001$). Pure strain females spawned in less than 35% of experiments, whereas hybrid females spawned in over 85% of experiments. Pure strain females spawned in front of a different species only when a conspecific male was not present. Hybrid females showed no apparent preference between the three types of male, and spawned in all experiments where they were given a choice of the same type of male.

Table 2.2 Frequency of spawning within ten days by the three types of female, with each type of male, during the initial mate choice experiments.

Female	Male spawned with (out of three tests for each pair of males)									Total no. of spawnings
	Between OSPIL	OMOSS	Between OSPIL	HYB.	Between OMOSS	HYB.	Both OSPIL	Both OMOSS	Both HYB.	
OMOSS	0	0	1	0	2	0	1	1	0	5 / 18
OSPIL	2	0	0	0	0	1	1	1	1	6 / 18
HYBRID	1	1	1	1	1	2	3	3	3	16 / 18

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus;* HYB., hybrid.

In the additional 72 mate choice experiments, the choice of mate was not random in any of the female types ($X^2 = 23.10$, $df = 4$, $P < 0.001$). Pure-bred females always spawned with a conspecific male when the other male was a heterospecific, although both species chose a hybrid male over a conspecific male in one instance (Table 2.3). *O. mossambicus* females spawned with a hybrid male, in preference to a heterospecific, whereas, *O. spilurus* females showed no preference

Table 2.3 Frequency of spawning by the three types of female, with each type of male during the additional mate choice experiments (unlimited time period).

Female	Male spawned with (eight tests for each pair)					
	Between OSPIL	OMOSS	Between OSPIL	HYB.	Between OMOSS	HYB.
OMOSS	0	8	2	6	7	1
OSPIL	8	0	7	1	4	4
HYBRID	1	7	2	6	6	2

OMOSS, *O. mossambicus;* OSPIL, *O.* spilurus; HYB., Hybrid.

between hybrid and heterospecific males. Hybrid females showed a preference for *O. mossambicus* males, over both *O. spilurus* and hybrid males. Hybrid females rarely chose to spawn with an *O. spilurus* male. The combined results from all mate-choice tests are summarised in Table 2.4.

Table 2.4 Results and findings from two-way contingency tests (G-statistic and probability) on mate choice data from the combined results of all mate choice experiments.

Mate choice tests		G-statistic	*P**	Summary of findings on mate choice between male types by females
Females	Males			
Pure-bred	Pure-bred	22.77	<0.001	OSPIL and OMOSS mate assortatively.
Pure-bred	Conspecific & HYBRID	0.02	n.s	Pure-bred females do not differ in preference - both prefer conspecific over HYBRID males.
Pure-bred	Heterospecific & HYBRID	0.21	n.s	Pure-bred females do not differ in preference (although OMOSS females showed a preference for HYBRID over OSPIL males).
OSPIL & HYBRID	OSPIL & HYBRID	5.97	0.05	OSPIL females prefer OSPIL over HYBRID males, HYBRID females prefer HYBRID over OSPIL males.
OMOSS & HYBRID	OMOSS & HYBRID	1.92	n.s	OMOSS and HYBRID females do not differ in preference - both prefer OMOSS over HYBRID males.
OSPIL & HYBRID	Pure-bred	30.29	<0.001	OSPIL females prefer OSPIL over OMOSS males, HYBRID females prefer OMOSS over OSPIL males.
OMOSS & HYBRID	Pure-bred	2.29	n.s	OMOSS and HYBRID females do not differ in preference - both prefer OMOSS over OSPIL males.
HYBRID	Pure-bred & HYBRID	2.26	n.s	Hybrid females do not prefer different pure-bred males (OSPIL or OMOSS) over hybrid males.

OMOSS, *O. mossambicus;* OSPIL, *O.* spilurus; * In all tests $df = 1$; n.s, not significant ($P>0.05$).

The choice of mate did not appear to be influenced by the presence of a nest. In almost all experiments (77.8%) both males had a nest, although the size of nest between males was not noted. Of the remaining tests, seven females spawned with males which did not have a nest (when the other male did), and nine females spawned with males which had a nest (when the other male did not). Of these tests, the choice of mate followed the general pattern outlined above. In tests where the same individual males had to be used again (due to a shortage of fish), no one male was spawned with more frequently than another.

The number of days taken to spawn, across all additional experiments, did not vary for the three types of female (Kruskal-Wallis test; $X^2 = 1.57$, $df = 2$, $P = 0.46$). Hybrid females took less time to spawn when the choice was between two pure-bred males, although this was not significantly different from the other tests. Both pure-bred females took longer to spawn when they

did not have the choice of a conspecific male (Table 2.5). The number of days that *O. spilurus* females took to spawn when given the choice of a hybrid or *O. mossambicus* male, was significantly longer than the time taken to spawn when one of the choices was a conspecific male. In this case, it took up to 34 days for the *O. spilurus* female to spawn.

Table 2.5 Mean number of days (± SE) taken to spawn by the three types of female, with each pair of males, during the additional mate choice experiments. Results of Kruskal-Wallis test on the number of days taken to spawn with each combination of males for each type of female.

Female	Mean number of days to spawn out of 8 tests (minimum - maximum)				
	Males OSPIL & OMOSS	Males OSPIL & HYBRID	Males OMOSS & HYBRID	X^2	*P*
OMOSS	9.87 ± 2.62 (2-19)	11.87 ± 4.08 (1-32)	5.37 ± 1.36 (1-13)	1.16	0.560
OSPIL	7.25 ± 3.76 (1-33)	9.25 ± 2.25 (1-18)	17.5 ± 3.56 (7-34)	7.53	0.023
HYBRID	9.12 ± 2.37 (2-24)	11.5 ± 3.66 (1-29)	10.5 ± 1.59 (4-18)	1.24	0.054

2.3.1.2 Visual contact between fish only (mate choice in pure-bred fish)

Of the seven mate choice experiments conducted where the fish were in separate tanks, spawning occurred in five experiments within 15 days. The remaining two experiments (one for each type of female) were abandoned after the female showed no intention of spawning after six weeks, when the fish no longer interacted with each other. When given the choice between a conspecific and a heterospecific male, *O. spilurus* females spawned in front of *O. spilurus* males on two occasions (3 days and 12 days taken to spawn), and in front of an *O. mossambicus* male in the remaining test (15 days to spawn). When given the choice between the two types of male, the *O. mossambicus* female spawned with a conspecific male in one test (4 days to spawn). In the other test, the choice of mate by the *O. mossambicus* female (9 days to spawn) could not be determined because nests had been constructed in front of both *O. spilurus* and *O. mossambicus* males.

2.3.2 Allocation of time that females spent in front of males

In both experiments (Forced-choice and Open-choice), all female types spent a similar amount of time in front of males, and no one male type had more or less time spent with it (i.e. the type of female and the type of male were not significant factors; See Table 2.7 and 2.9).

2.3.2.1 Forced-choice experiments - time allocation in front and away from males

Across all Forced-choice experiments, females spent significantly more time in front of the male than away from the male behind the central partition (mean time ± SE in seconds: 741.3 ±

Table 2.6 Mean time (± SE, minimum - maximum times) that females spent with each male type (nine replicates of each test) in Forced-choice experiments (900 s long).

Female type	Mean time (s) with all males	Mean time (s) with each male type		
		OMOSS	OSPIL	HYBRID
OMOSS	783.5 ± 25.0	822.6 ± 31.4 (654 - 900)	801.3 ± 37.3 (611 - 900)	725.4 ± 54.9 (460 - 900)
OSPIL	711.2 ± 37.2	619.7 ± 78.5 (152 - 900)	750.0 ± 54.3 (441 - 900)	764.0 ± 53.1 (374 - 900)
HYBRID	729.2 ± 34.4	798.8 ± 51.2 (503 - 900)	665.0 ± 62.9 (381 - 893)	723.8 ± 62.1 (358 - 900)

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

18.9 and 158.7 ± 18.9, respectively; Wilcoxon signed ranks tests, $z = -7.54$, $P<0.001$), although in one case an *O. spilurus* female spent only 152 s, of the 900 s observation period, in front of an *O. mossambicus* male (Table 2.6). The time that females spent with the first and the second male, presented to them, did not differ (797.4 ± 31.6 and 749.0 ± 32.7, respectively; Wilcoxon signed ranks tests, $z = -1.06$, $P = 0.29$). However, less time was spent with both males if they were of the same type, compared to experiments where the two males shown consecutively were of a different type (677.5 ± 30.9 and 773.2 ± 22.7, respectively; Mann-Whitney U-test, $z = -3.06$, $P = 0.002$). When the two males were of the same type, females appeared to occasionally leave the male being shown and 'search' for the other male (hidden from view by a partition). Across all experiments, pure-bred females spent slightly more time in front of conspecific males than in front of non-conspecific males (Table 2.6), although time allocation was not significantly affected by the type of male and female being tested (Table 2.7).

Table 2.7 Two-way ANOVA (*F*-ratio and probability) of the time females spent in front of males during the 15 minute Forced-choice experiments.

	df	*F*-ratio	*P*
Female type	2	1.030	0.362
Male type	2	0.147	0.863
Female*Male	4	1.919	0.117
Residuals	72		

2.3.2.2 Open-choice experiments - time allocation between two males

In Open-choice experiments, time allocation was affected by the type of male and female being tested together, and by the type of female and type of other male present (Table 2.8 and 2.9).

A. Interaction between male type and female type

All three types of female spent most time with their own males overall. Pure-bred females

Table 2.8 Mean time (seconds ± SE) that each female type spent in front of each type of male (irrespective of other male present) across all experiments (nine replicates), and the mean time that each female type spent in front of each type of male with each type of other male present (three replicates of each), during the 30 minute Open-choice tests.

Female type	Male type	Mean time with male irrespective of O.m.p	Mean time with male when Other male present is: OMOSS	OSPIL	HYBRID
OMOSS	OMOSS	978.8 ± 99.7	815.7 ± 107.4	1183.0 ± 140.7	937.7 ± 230.4
	OSPIL	632.3 ± 68.7	522.0 ± 129.5	650.0 ± 85.7	725.0 ± 148.2
	HYBRID	748.1 ± 126.5	738.3 ± 235.4	1005.7 ± 151.6	500.3 ± 222.7
OSPIL	OMOSS	654.2 ± 99.3	858.3 ± 182.7	473.0 ± 27.6	631.3 ± 215.9
	OSPIL	928.8 ± 97.3	1159.3 ± 17.3	612.3 ± 234.0	1014.7 ± 121.1
	HYBRID	753.7 ± 107.6	1039.3 ± 163.1	685.0 ± 146.4	536.7 ± 155.6
HYBRID	OMOSS	793.7 ± 117.8	864.7 ± 264.4	918.7 ± 177.0	597.7 ± 189.0
	OSPIL	729.8 ± 87.3	648.0 ± 153.2	984.7 ± 77.8	556.7 ± 106.0
	HYBRID	956.6 ± 122.3	1042.3 ± 154.4	1040.3 ± 223.7	787.0 ± 502.0

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus;* O.m.p, Other male present.

spent more time in front of conspecific males, least time in front of non-conspecifics and an intermediate mean time in front of hybrid males. Hybrid females spent more time in front of hybrid males, and showed a preference for *O. mossambicus* males over *O. spilurus* males (Table 2.8 and Figure 2.4). *O. spilurus* females spent significantly more time with *O. spilurus* males than with *O. mossambicus* males (t-value = −15.45, $P<0.005$), but there were no other significant results in the pair-wise comparisons (Table I.1, Appendix I). Results of G-tests, based on counts of males with which the longest time was spent (see Table I.1, Appendix I) and a summary of findings, are presented in Table 2.10. These results largely reflect the final choice of mate with which the female spawned, apart from the preference of hybrid females between hybrid and *O. mossambicus* males (Table 2.4). Hybrid females spent more time with hybrid over *O. mossambicus* males, whereas, hybrid females chose to spawn with *O. mossambicus* over hybrid males.

Table 2.9 Three-way ANOVA of the time females spent in front of males during the 30 minute Open-choice experiments.

	df	*F*-ratio	*P*
Female type	2	0.206	0.815
Male type	2	0.274	0.762
O.m.p type	2	2.296	0.110
Female*Male	4	3.190	0.020
Female*O.m.p	4	3.819	0.008
Male*O.m.p	4	0.908	0.466
Female*Male*O.m.p	8	0.487	0.860
Residuals	54		

O.m.p; other male type present when the time the female spent in front of the male was recorded.

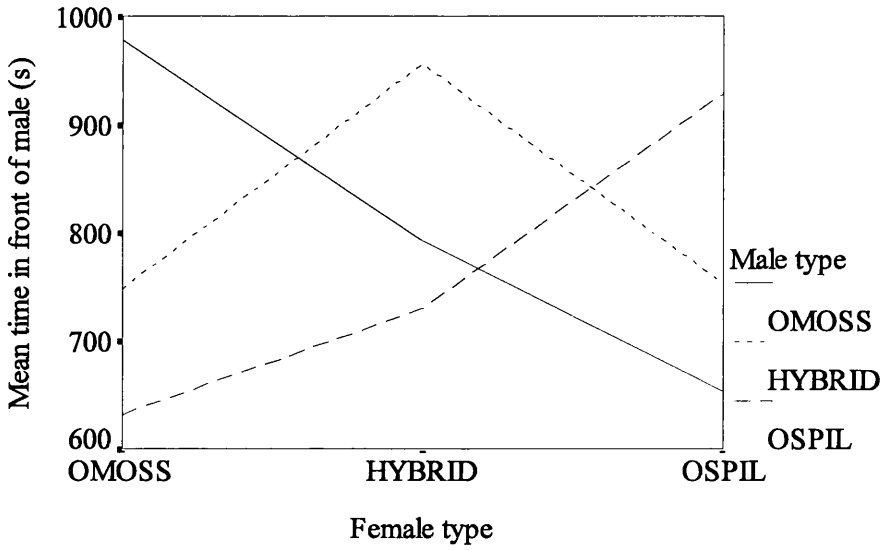


Figure 2.4 The mean time that *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid females spent in front of each type of male (irrespective of the other male present), during the 30 minute Open-choice experiments. See Table 2.8 for standard error of means.

Table 2.10 Results of two-way contingency tests (G-statistic and probability) on time allocation between males by female (based on 'longest time' results in Table I.1, Appendix I), and summary of findings on female preference between male types (i.e. male with which female spent most time with).

Mate choice tests		G-statistic	*P**	Summary of findings on time allocation between male types by each female type
Females	Males			
Pure-bred	Pure-bred	6.65	0.01	OSPIL and OMOSS prefer conspecific males.
Pure-bred	Conspecific & HYBRID	0.00	n.s	Pure-bred females do not differ in preference - both prefer conspecific over HYBRID males.
Pure-bred	Heterospecific & HYBRID	1.05	n.s	Pure-bred females do not differ in preference - both prefer HYBRID over heterospecific males.
OSPIL & HYBRID	OSPIL & HYBRID	2.96	n.s	OSPIL and HYBRID females do not differ in preference (although HYBRID females generally spent more time with HYBRID males than with OSPIL males).
OMOSS & HYBRID	OMOSS & HYBRID	0.54	n.s	OMOSS and HYBRID females do not differ in preference - both show no difference in time allocation between OMOSS and HYBRID males.
OSPIL & HYBRID	Pure-bred	6.65	0.01	OSPIL females prefer OSPIL over OMOSS males, and HYBRID females prefer OMOSS over OSPIL males.
OMOSS & HYBRID	Pure-bred	0.00	n.s	OMOSS and HYBRID females do not differ in preference - both prefer OMOSS over OSPIL males.
HYBRID	Pure-bred & HYBRID	1.05	n.s	Hybrid females do not prefer different pure-bred males (OSPIL or OMOSS) over hybrid males.

OMOSS, *O. mossambicus;* OSPIL, *O.* spilurus; * In all tests $df = 1$; n.s, not significant.

B. Interaction between female type and other male present (O.m.p) type

Across all tests, all female types spent least time with the male when the other male present (O.m.p) was the same type as the female (Table 2.11 and Figure 2.5). Pure-bred females spent most time with hybrid males when the O.m.p was a heterospecific. Hybrid females spent least time with any male when the O.m.p was a hybrid. Hybrid females spent slightly more time with a male if the O.m.p was *O. spilurus* compared to when it was *O. mossambicus* (Table 2.8). As in Forced-choice tests, pure-bred females generally spent least time in front of the male when the two males were of the same type (Table 2.8).

Table 2.11 Mean time (seconds ± SE) that females spent with males (irrespective of male type) with each type of other male present (O.m.p), during Open-choice experiments (nine replicates of each).

Female type	Other male present (O.m.p)		
	OMOSS	OSPIL	HYBRID
OMOSS	692.0 ± 94.4	946.2 ± 101.6	721.0 ± 119.9
OSPIL	1019.0 ± 82.3	590.1 ± 65.9	727.6 ± 111.6
HYBRID	851.7 ± 114.1	981.2 ± 87.1	647.1 ± 110.3

OMOSS, *O. mossambicus;* OSPIL, *O.* spilurus

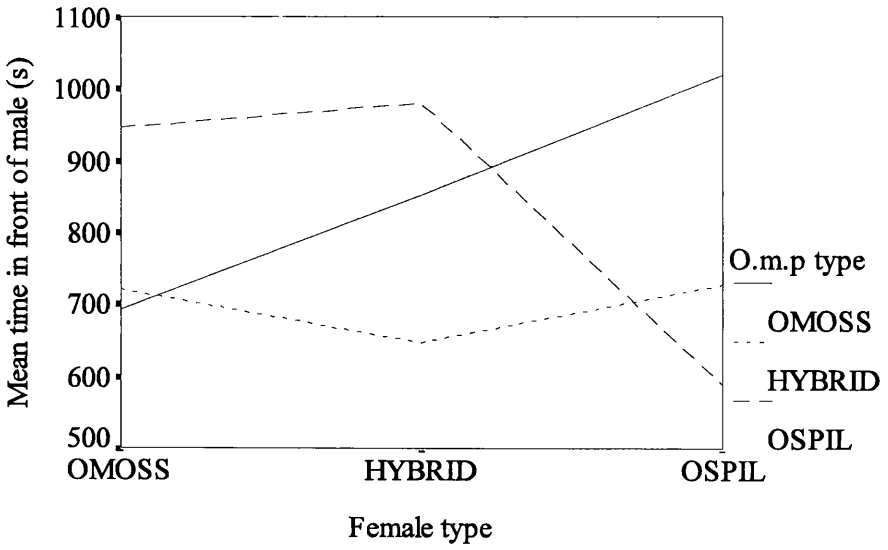


Figure 2.5 The mean time that *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid females spent in front of males (irrespective of male type) with each type of other male present (O.m.p), during the 30 minute Open-choice experiments. See Table 2.8 for standard error of means.

2.3.3 Behaviour

2.3.3.1 General observations

No variation was noted in the form of behaviours between female types or between male types. However, *O. mossambicus* males were more timid than the other males and they were easily disturbed by the observer. Differences were observed in frequency of displays between sexes, although these were not tested statistically. Males performed more nods and shakes than females. Nodding was also more exaggerated in males (i.e. body held at a steeper angle) and, unlike females, often involved a 'leading' movement towards the nest and was followed by shaking behaviour. Females performed more frontal displays than males, but the frequency of lateral displays did not vary between sexes. Across all types of fish, in both Open-choice and Forced-choice experiments, no one sex consistently performed more or less digs and bites.

The number of displays varied greatly between replicates and were often more variable in males. For both sexes, the number of displays performed by hybrid fish varied more between experiments than those performed by pure-bred fish. Biting and digging were the most common, and often the most variable, behaviours observed. Quivering and skimming were rarely performed by any type of fish, of both sexes (mean less than 1 display per experiment, median = 0), and were therefore not considered for statistical analyses. Quivering was more frequently performed by males, than by females, both in the number of experiments it occurred in, and in the number of displays performed. *O. mossambicus* males performed more 'quivers' than the other male types, whereas hybrid females performed more quivers than the other types of female. The frequency of skimming behaviour did not vary between the three types of fish. Skimming was more frequently performed by females (i.e. in the number of experiments where skimming occurred), although both sexes performed a similar number of 'skims' during any single experiment.

The frequency of displays for female behaviours was generally greatest in hybrids and similar in pure-bred females. For some behaviours, the frequency of displays performed by hybrid females resembled those of *O. spilurus* more than those of *O. mossambicus* females. For male behaviours, the frequency of displays was generally greatest in hybrids and similar in, or varied between, pure-bred males. The number of displays performed was also affected by the type of fish being tested together. These results (sections 2.3.3.2 and 2.3.3.3) are related to whether differences in display rate can predict the final choice of spawning partner. A comparatively greater number of displays by males and females was interpreted as a preference for a particular fish. Results of Open-and Forced-choice experiments did not differ, and are therefore presented together.

2.3.3.2 Female behaviour

The mean number of displays performed by females, for six behaviours, in Forced-choice experiments are presented in Table I.2 (Appendix I). The mean number of displays performed by females to pairs of males in Open-choice experiments are presented in Table 2.16 (tests where the two male types differed) and in Table I.3 (tests where the two male types were the same). The mean number of displays performed by the three types of females to each type of male, across all Open-choice experiments, are presented in Table I.4 (Appendix I).

In both experiments (Forced-choice and Open-choice) there was no variation in the number of displays that all females performed in front of the three types of male (i.e. male type was not a significant factor; Table 2.12 and Table 2.13). In both experiments, the frequency of displays varied significantly between the three types of female, for individual behaviours and across all behaviours (i.e. female type was a significant factor; see *Section A*). There were few statistically significant interactions between the type of female and type of male being tested. However, trends were observed in the frequency of displays between different combinations of female and male types. Differences in the number of displays performed by females to male types are presented with reference to spawning partner preference (*Section B*).

In Forced-choice experiments the frequency of displays was also affected by the two types of male that were consecutively presented to the female. Similarly, there was a highly significant interaction between the two types of male being tested (male and O.m.p) in Open-choice experiments (*Section C*). Female behaviour was not generally affected by the type of other male present (Table 2.13).

The number of frontal displays

Table 2.12 Two-way ANOVA on the number of displays recorded for females, for six behaviours individually and for the multivariate analysis of all behaviours, during Forced-choice experiments.

Beh.		Female type	Male type	Female *male
Uni.*		(2 *df*)	(2 *df*)	(4 *df*)
Bi.	*F*	4.683	2.861	0.081
	P	0.012	0.064	0.988
Di.	*F*	9.811	2.072	1.098
	P	<0.001	0.133	0.364
F.D.	*F*	1.091	1.207	0.178
	P	0.341	0.305	0.949
L.D.	*F*	0.101	1.508	0.356
	P	0.904	0.228	0.839
No.	*F*	1.013	0.825	0.784
	P	0.368	0.442	0.539
Sh.	*F*	1.709	0.241	2.243
	P	0.188	0.786	0.073
Multi.†		(12 *df*)	(12 *df*)	(24 *df*)
All Beh.	*F*	2.845	1.018	0.763
	P	0.002	0.436	0.781

Beh., see Table 2.1 for description of behaviours.
* Univariate ANOVA: Residual = 72 *df*
† Multivariate ANOVA: Error of Female type and Male type = 134.0 *df*; Error of Male*Female = 234.95 *df*

performed was highly dependent on all three types of fish being tested (Table 2.13). A significant three-way interaction was also observed when all behaviours were considered (multivariate analysis); females differed in behaviour to particular males under different choice conditions.

Table 2.13 Three-way ANOVA (*F*-ratio and probability) on the number of displays recorded for females, for six behaviours individually and for the multivariate analysis of all six behaviours, during the Open-choice experiment.

Behaviour		Female type	Male type	O.m.p type	Female *Male	Female *O.m.p	Male *O.m.p	Female*Male *O.m.p
Univariate †		(2 *df*)	(2 *df*)	(2 *df*)	(4 *df*)	(4 *df*)	(4 *df*)	(8 *df*)
Biting	*F*	1.032	1.548	0.854	1.151	0.560	2.572	1.081
	P	0.363	0.222	0.431	0.343	0.693	0.048 ♯	0.390
Digging	*F*	3.724	0.114	0.127	0.303	0.746	0.646	1.137
	P	0.031 ♯	0.893	0.881	0.874	0.565	0.632	0.354
Frontal display	*F*	5.513	0.130	0.178	0.583	1.171	25.110	4.184
	P	0.007	0.878	0.837	0.677	0.334	<0.001	0.001
Lateral display	*F*	0.038	1.034	0.471	0.861	1.401	6.893	1.186
	P	0.962	0.363	0.627	0.494	0.246	<0.001	0.324
Nodding	*F*	6.563	0.444	1.047	3.785	1.859	4.566	1.738
	P	0.003	0.644	0.358	0.009	0.131	0.003	0.111
Shaking	*F*	0.869	0.922	4.057	1.724	1.487	0.667	0.938
	P	0.425	0.404	0.023	0.158	0.219	0.618	0.493
Multivariate *		(12 *df*)	(12 *df*)	(12 *df*)	(24 *df*)	(24 *df*)	(24 *df*)	(48 *df*)
All Beh.	*F*	3.236	0.649	1.427	1.134	1.143	3.957	1.497
	P	0.001	0.795	0.166	0.143	0.303	<0.001	0.026

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus;* O.m.p, Other male present. Behaviour, see Table 2.1 for description of behaviours. ♯ Not significant at the 5% level when the Bonferroni procedure is applied.† Univariate ANOVA: Residual = 54 *df.* * Multivariate ANOVA: Error of Female type, Male type and O.m.p type = 98 *df*; Error of Female*Male, Female*O.m.p and Male*O.m.p = 172.15 *df*; Error of Male*Female*O.m.p = 245.16 *df.*

A. Differences in the number of displays given by female types

In both Forced- and Open-choice experiments, across all six behaviours, the frequency of displays differed between types of female (Table 2.12 and 2.13). Lateral display was the only behaviour found not to vary between female types (statistically significant or not). Biting and digging behaviour was far more common in hybrid females than in pure-bred females (Figure 2.6, Table 2.14 and Table 2.15). In Forced-choice experiments, hybrid females also performed more displays than pure-bred females for all other behaviours except nodding (Table 2.14). In both experiments, the mean frequency of nodding behaviour by hybrid females resembled that of *O. spilurus* females. In Open-choice experiments hybrid females performed a similar number of

frontal displays to *O. spilurus* females, whereas the frequency of shaking resembled that of *O. mossambicus* females (Table 2.15). In both experiments, pure-bred females did not differ in the number of bites or digs they performed. However, differences were observed between pure-bred females in the frequency of frontal displays, nods and shakes. *O. mossambicus* females performed fewer shakes than *O. spilurus* females. *O. mossambicus* females also performed fewer frontal displays and more nods (Figure 2.7) than *O. spilurus* or hybrid females (Tables 2.14 and 2.15). In general, hybrids were more aggressive than pure-bred females, and *O. spilurus* more aggressive than *O. mossambicus*.

Table 2.14 Mean number of displays (± SE, of 27 tests) for six behaviours, performed by each type of female during Forced-choice experiments.

Beh.	Female type		
	OMOSS	OSPIL	HYBRID
Bi.	21.0 ± 6.2	17.5 ± 5.2	46.8 ± 10.9
Di.	6.1 ± 2.2	5.9 ± 2.1	21.4 ± 4.5
F.D.	7.9 ± 1.8	12.2 ± 2.2	10.0 ± 2.1
L.D.	11.8 ± 1.4	11.3 ± 1.4	12.3 ± 2.1
No.	2.6 ± 0.6	1.7 ± 0.4	1.7 ± 0.6
Sh.	1.5 ± 0.6	1.1 ± 0.3	2.6 ± 0.8

Table 2.15 Mean number of displays (± SE, of 27 tests) for six behaviours, performed by each type of female during Open-choice experiments.

Beh.	Female type		
	OMOSS	OSPIL	HYBRID
Bi.	17.1 ± 4.8	25.0 ± 9.0	45.3 ± 10.8
Di.	14.3 ± 4.2	11.9 ± 2.0	32.7 ± 5.5
F.D.	6.6 ± 1.9	12.8 ± 1.8	12.9 ± 3.3
L.D.	13.2 ± 1.8	13.8 ± 2.0	13.2 ± 2.2
No.	3.8 ± 0.7	1.6 ± 0.5	1.6 ± 0.5
Sh.	1.4 ± 0.4	0.9 ± 0.3	1.6 ± 0.5

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.* Beh., see Table 2.1 for description of behaviours.

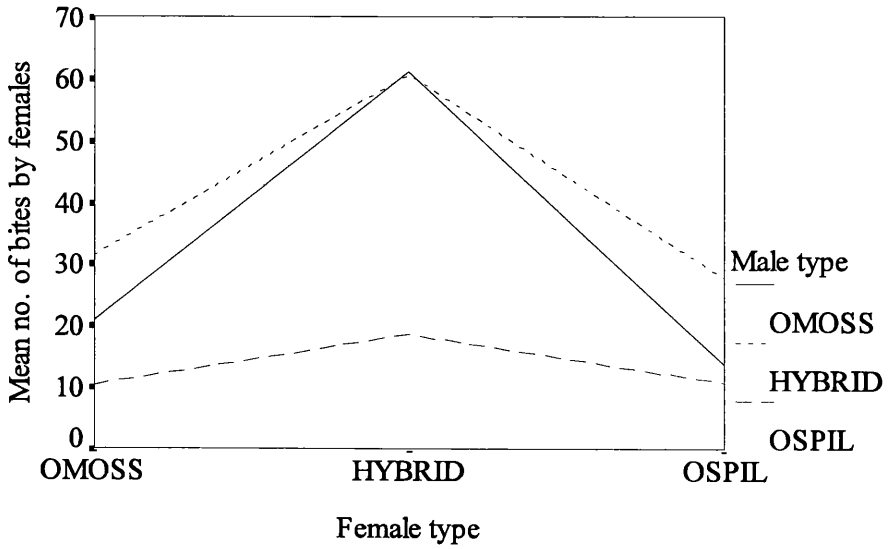


Figure 2.6 Mean number of bites performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid females in front of each type of male during the 15 minute Forced-choice experiments. See Table I.2 (Appendix I) for standard error of means.

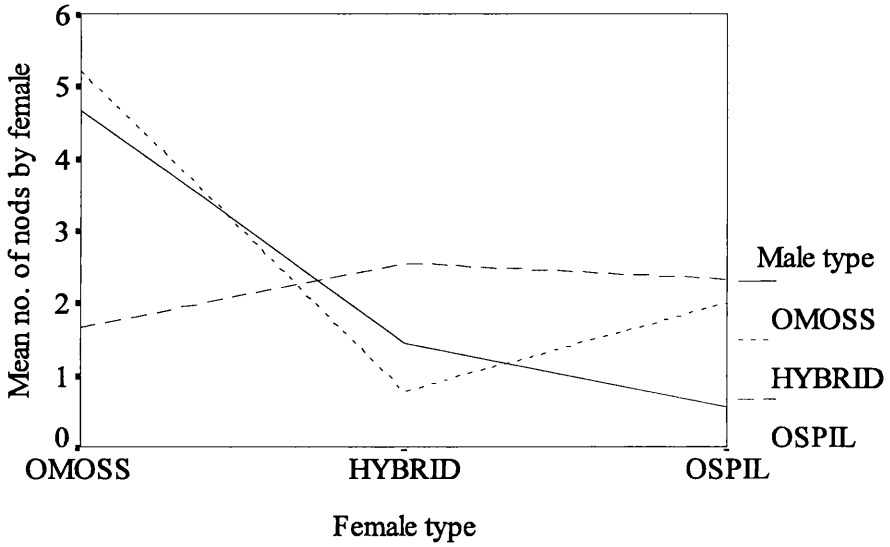


Figure 2.7 Mean number of nods performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid females in front of each type of male during the 30 minute Open-choice experiments. See Table I.4 (Appendix I) for standard error of means.

B. The effect of male and female type on female behaviour - Predicting mate preference

In Open-choice experiments, where females were presented with two different male types, female behaviour differed significantly between pairs of males in 18 of 27 tests (three replicates of nine different tests). Significant differences were found in at least two of the three replicate tests for all combinations of fish, except in tests where hybrid females were given the choice of pure-bred males (Table 2.16). The frequency of nodding during Open-choice experiments, was the only case in which there was a statistically significant interaction between the type of female and the type of male being tested (Table 2.13). Over all Open-choice experiments pure-bred females performed fewer nods in front of heterospecific males than in front of conspecific and hybrid males (Figure 2.7). This trend was observed across most behaviours. Pure-bred females performed a similar number of nods to hybrid and conspecific males, and hybrid females performed more nods to pure-bred males than to hybrid males (Figure 2.7 and Table I.4). The number of displays performed to hybrid males, and by hybrid females, varied between experiments and behaviours.

The following trends were observed in the behaviour of female types to different males types when the results, from Forced-choice experiments (Table I.2, Appendix I), across all Open-choice experiments (Table I.4, Appendix I) and from comparisons between paired males in Open-choice tests (Table 2.16), were compiled:

1) *Pure-bred females to pure-bred males* - In both experiments, *O. mossambicus* females generally performed more displays in front of conspecifics than in front of heterospecifics. In Forced-choice

experiments, there was no consistent pattern in the number of displays performed by *O. spilurus* females for each behaviour to pure-bred males (Table I.2). Across all Open-choice experiments, *O. spilurus* females generally performed more displays in front of *O. spilurus* than in front of *O. mossambicus* males (Table I.4). When the number of displays given to paired males are compared from tests where *O. spilurus* females were given the choice of pure-bred males, more displays were performed in front of *O. spilurus* males for all behaviours (Table 2.16). These results predict spawning preference, in that, pure-bred females prefer conspecific over heterospecific males.

2) *Pure-bred females to conspecific and hybrid males* - In Forced-choice experiments, pure-bred females performed more displays to hybrid males than to conspecific males, except nodding and

Table 2.16 Mean (± SE) number of displays performed by females, for six behaviours recorded during Open-choice experiments, in tests where the two males presented to the female were of different types (three replicates of each test), and results of R X C Tests of Independence using *G*-test* (Means for tests where two males were the same, in Table I.3 Appendix I).

Female	Beh.	Male types presented to female					
		OMOSS & OSPIL		OMOSS & HYBRID		OSPIL & HYBRID	
OMOSS	Bi.	18.7 ± 9.1	3.3 ± 1.8	15.3 ± 4.8	45.7 ± 36.1	7.0 ± 1.1	35.0 ± 20.1
	Di.	5.7 ± 2.3	16.0 ± 8.2	39.0 ± 31.0	1.7 ± 0.9	9.7 ± 6.1	13.0 ± 6.6
	F.D.	1.3 ± 0.9	0.0 ± 0.3	2.7 ± 2.7	0.7 ± 0.7	2.3 ± 1.4	10.0 ± 4.3
	L.D.	10.3 ± 4.8	3.0 ± 1.7	13.3 ± 0.9	12.3 ± 7.8	11.3 ± 5.6	16.3 ± 5.4
	No.	1.0 ± 1.0	0.0 ± 0.0	7.3 ± 2.3	7.0 ± 3.6	2.7 ± 2.7	4.7 ± 0.7
	Sh.	4.3 ± 2.6	1.3 ± 0.7	1.7 ± 0.9	0.7 ± 0.7	1.3 ± 0.7	2.3 ± 1.2
Significant G-tests		(2) 23.76<*G*<26.17		(2) 30.63<*G*<73.24		(2) 16.94<*G*<28.87	
OSPIL	Bi.	5.3 ± 3.9	10.3 ± 7.5	12.3 ± 5.9	5.7 ± 1.8	7.3 ± 2.3	32.3 ± 13.6
	Di.	14.7 ± 8.8	15.7 ± 12.7	13.3 ± 5.4	15.7 ± 7.0	10.0 ± 1.0	11.7 ± 4.2
	F.D.	3.3 ± 2.0	11.0 ± 6.1	9.7 ± 4.3	12.7 ± 2.7	8.0 ± 0.6	10.7 ± 2.2
	L.D.	2.7 ± 0.7	15.3 ± 7.5	11.3 ± 6.4	13.0 ± 3.0	11.7 ± 1.4	12.0 ± 3.2
	No.	1.0 ± 1.0	2.0 ± 2.0	0.0 ± 0.0	5.0 ± 1.5	12.3 ± 0.7	1.0 ± 0.6
	Sh.	0.3 ± 0.3	3.3 ± 2.0	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0	1.3 ± 0.9
Significant G-tests		(2) 15.65<*G*<18.99		(2) 14.41<*G*<22.57		(2) 15.12<*G*<15.96	
HYBRID	Bi.	21.0 ± 15.5	8.3 ± 1.8	21.7 ± 17.7	14.0 ± 14.0	9.3 ± 5.9	61.7 ± 29.8
	Di.	35.0 ± 17.4	26.7 ± 17.1	29.3 ± 19.6	62.0 ± 19.7	38.7 ± 5.8	18.3 ± 8.2
	F.D.	0.0 ± 0.0	0.7 ± 0.7	0.0 ± 0.0	0.3 ± 0.3	5.0 ± 1.0	5.3 ± 1.4
	L.D.	4.4 ± 1.8	3.0 ± 3.0	4.7 ± 1.4	8.3 ± 6.9	8.7 ± 3.3	18.0 ± 7.5
	No.	0.7 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.0	1.0 ± 1.0
	Sh.	2.3 ± 1.3	1.3 ± 1.3	0.7 ± 0.7	5.0 ± 2.4	0.0 ± 0.0	1.0 ± 1.0
Significant G-tests		(1) *G* = 20.52		(2) 14.77<*G*<85.41		(3) 18.50<*G*<53.23	

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.* Beh., see Table 2.1 for description of behaviours.
**Significant G-tests* - Number of tests out of three, where the number of female displays performed (across all behaviours) are significantly different between paired males (in parenthesis), and all significant *G* statistics where *df* = 5, and when *G*= 11.07, *P* = 0.05; when *G*= 15.09, *P* = 0.01; and when *G*= 20.52, *P* = 0.001.

shaking (Table I.2). Across all Open-choice experiments, the behaviour of pure-bred females to conspecific and hybrid males varied, although more bites were performed in front of hybrid males (Table I.4). When the number of displays given to paired males are compared from tests where pure-bred females were given the choice of conspecific and hybrid males, the pattern of behaviour differed between female types (Table 2.16). *O. mossambicus* females performed more displays to conspecific males than to hybrid males, except biting (an aggressive behaviour). *O. spilurus* females performed more displays to hybrid males than to conspecifics, except nodding (a sexual behaviour). A high frequency of displays did not always predict mate preference.

3) *Pure-bred females to heterospecific and hybrid males* - In both experiments, pure-bred females generally performed more displays in front of hybrid than in front of heterospecific males. These results predict the spawning preference of pure-bred females for hybrid over heterospecific males.

4) *Hybrid females to all male types* - In Forced-choice experiments and across all Open-choice experiments, there was no clear pattern in the behaviour of hybrid females to each type of male. When the number of displays are compared from Open-choice tests where hybrid females were given the choice of pure-bred males, hybrid females generally performed more to *O. mossambicus* than to *O. spilurus* males, although behaviours differed significantly in only one of the three G-tests (Table 2.16). When given a choice of hybrid and pure-bred males, the behaviour of hybrid females varied with different types of pure-bred male, although hybrid females generally performed more to hybrid males than to pure-bred males (Table 2.16).

C. *Interactions between the two males presented to the female*

In Forced-choice experiments, the number of displays often increased when the female was shown the same type of male consecutively; this was despite the decrease in time spent with both males (section 2.3.2.1). A significant increase in displays was observed for the behaviours frontal display, lateral display and nodding (Table I.5 and Figure I.1, Appendix I). Similarly, when females were presented with males of the same type in Open-choice experiments the three aggressive behaviours recorded (biting, frontal display and lateral display) all increased. For example, the number of frontal displays performed in front of an *O. mossambicus* male were greater if the other male present was also an *O. mossambicus* male compared to an *O. spilurus* or a hybrid male. In pure-bred males, the number of frontal displays was lowest if the other male present was a non-conspecific (Figure 2.8 and Table I.6). In general, the same interaction was observed when both males were hybrids.

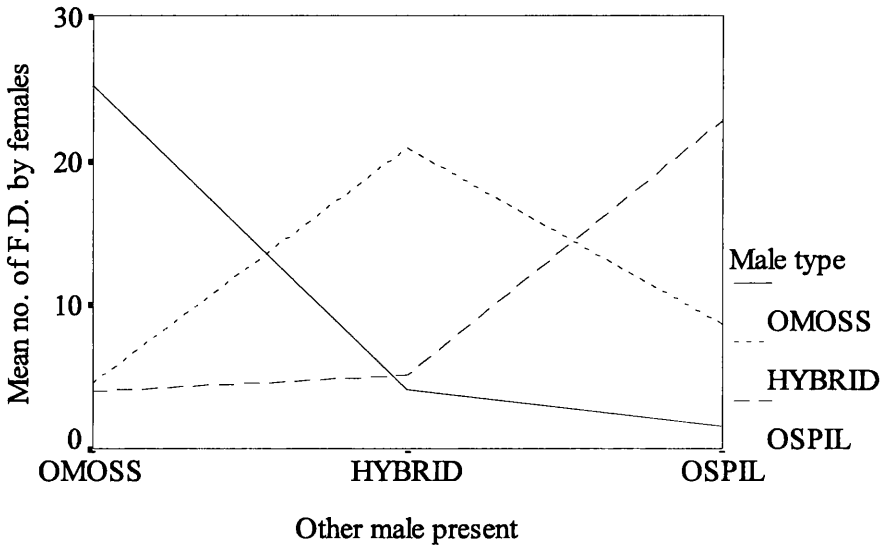


Figure 2.8 Mean number of frontal displays performed by all females in front of *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid males with each type of other male present, during 30 minute Open-choice experiments. See Table I.6 for standard error of means.

2.3.3.3 Male behaviour

The mean number of displays performed by males, for six behaviours, in Forced-choice experiments are presented in Table I.7 (Appendix I). The mean number of displays performed by pairs of males to females in Open-choice experiments are presented in Table 2.21 (tests where the two male types differed) and in Table I.8 (tests where the two male types were the same). The mean number of displays performed by the three types of males to each female type, across all Open-choice experiments, are presented in Table I.9 (Appendix I). Many of the patterns observed in male behaviour were similar to those observed for females.

In both experiments (Forced- and Open-choice), for the six behaviours recorded, there was little variation in the number of displays that all males performed

Table 2.17 Two-way ANOVA (univariate and multivariate analysis) on the number of displays recorded for males for six behaviours, during Forced-choice experiments.

Beh.		Female type	Male type	Female * male
Uni.*		(2 *df*)	(2 *df*)	(4 *df*)
Bi.	*F*	0.861	5.479	0.953
	P	0.427	0.006	0.439
Di.	*F*	0.285	1.900	1.492
	P	0.753	0.157	0.214
F.D.	*F*	0.410	7.150	0.312
	P	0.665	0.001	0.869
L.D.	*F*	1.733	0.727	0.992
	P	0.184	0.487	0.417
No.	*F*	0.257	0.154	1.226
	P	0.774	0.857	0.308
Sh.	*F*	1.064	3.081	3.129
	P	0.350	0.052	0.020
Multi. †		(12 *df*)	(12 *df*)	(24 *df*)
All Beh.	*F*	0.661	3.350	1.146
	P	0.785	<0.001	0.295

Beh., see Table 2.1 for description of behaviours.
* Univariate ANOVA: Residual = 72 *df*
† Multivariate ANOVA: Error of Female and Male type = 134.0 *df*; Error of Male*Female = 234.95 *df*

in front of the three types of female (Table 2.17 and 2.18). Males performed less lateral displays and more nods in front of hybrid females than in front of pure-bred females. In both experiments, the frequency of displays varied significantly between the three types of male, for individual behaviours and across all behaviours (*Section A*). Interactions between the type of female and type of male being tested were not generally statistically significant, although some trends were observed. Differences in the number of displays performed by males to female types are presented with reference to spawning partner preference (*Section B).*

In both experiments the frequency of displays was affected by the two types of male presented to the female (*Section C*). The interaction between the two types of male being tested (male and O.m.p) was highly significant (Table 2.18). The O.m.p was not a significant factor in Open-choice experiments, and male behaviours were not generally affected by the type of female and O.m.p being tested (*Section D*). Unlike female behaviour, male behaviour did not significantly differ under different choice conditions. Nodding was the only behaviour which was affected by all three fish present in the experiment (Table 2.18).

Table 2.18 Three-way ANOVA (univariate and multivariate analysis) on the number of displays recorded for males, for six behaviours, during Open-choice experiments.

Behaviour		Female type	Male type	O.m.p type	Female *Male	Female *O.m.p	Male *O.m.p	Female*Male *O.m.p
Univariate †		(2 *df*)	(2 *df*)	(2 *df*)	(4 *df*)	(4 *df*)	(4 *df*)	(8 *df*)
Biting	*F*	0.231	10.492	0.476	2.137	1.538	2.545	0.895
	P	0.794	<0.001	0.624	0.089	0.204	0.050 ♯	0.527
Digging	*F*	0.087	1.314	0.104	0.347	0.809	2.203	0.448
	P	0.916	0.277	0.902	0.845	0.525	0.081	0.887
Frontal display	*F*	1.108	13.449	0.311	1.458	2.916	4.096	1.718
	P	0.338	<0.001	0.734	0.228	0.030♯	0.006	0.115
Lateral display	*F*	4.985	1.058	0.448	1.482	1.430	1.359	0.716
	P	0.010	0.354	0.641	0.220	0.237	0.260	0.677
Nodding	*F*	3.181	2.370	0.516	0.869	0.827	6.986	2.760
	P	0.049♯	0.103	0.600	0.489	0.514	<0.001	0.012
Shaking	*F*	0.105	1.464	0.406	0.867	0.907	3.364	0.532
	P	0.900	0.240	0.668	0.490	0.467	0.016	0.827
Multivariate *		(12 *df*)	(12 *df*)	(12 *df*)	(24 *df*)	(24 *df*)	(24 *df*)	(48 *df*)
All Beh.	*F*	1.370	4.158	0.271	1.104	1.404	4.397	1.033
	P	0.193	<0.001	0.992	0.344	0.111	<0.001	0.423

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus;* O.m.p, Other male present. Behaviour, see Table 2.1 for description of behaviours. ♯ Not significant at the 5% level when the Bonferroni procedure is applied.
† Univariate ANOVA: Residual = 54 *df.* * Multivariate ANOVA: Error of Female, Male and O.m.p type = 98 *df*; Error of Female*Male, Female*O.m.p and Male*O.m.p = 172.15 *df*; Error of Male*Female*O.m.p = 245.16 *df.*

A. Differences in the number of displays given by male types

In both experiments, biting and digging were more common in hybrids than in pure-bred males (Figure 2.9, Table 2.19 and Table 2.20). In Forced-choice experiments, hybrid males also performed more displays than pure-bred males for all other behaviours except shaking (Table 2.19). In Open-choice experiments hybrid males performed a similar number of frontal displays and lateral displays to *O. spilurus* males (Table 2.20). In both experiments, *O. spilurus* males performed more bites (Figure 2.9) and frontal displays (Figure 2.10) than *O. mossambicus* males. *O. spilurus* males performed more shakes than both other male types (Figure 2.11). *O. mossambicus* males performed more digs than *O. spilurus* males. In general, hybrids were more aggressive than pure-bred males, and *O. spilurus* more aggressive than *O. mossambicus*.

Table 2.19 Mean number of displays (± SE of 27 tests), for six behaviours, performed by males during Forced-choice experiments.

Beh.	Male type		
	OMOSS	OSPIL	HYBRID
Bi.	13.1 ± 5.3	17.1 ± 5.1	56.4 ± 13.8
Di.	18.8 ± 2.9	11.2 ± 3.1	20.0 ± 3.4
F.D.	1.8 ± 0.5	6.1 ± 1.0	8.0 ± 1.6
L.D.	10.1 ± 1.6	9.5 ± 1.1	12.4 ± 1.7
No.	17.0 ± 3.7	17.9 ± 3.2	18.3 ± 4.6
Sh.	4.8 ± 0.7	8.4 ± 1.4	4.5 ± 1.0

Table 2.20 Mean number of displays (± SE of 27 tests), for six behaviours, performed by males during Open-choice experiments.

Beh.	Male type		
	OMOSS	OSPIL	HYBRID
Bi.	16.4 ± 5.6	40.1 ± 19.5	86.8 ± 16.7
Di.	12.4 ± 2.5	5.2 ± 1.1	21.6 ± 5.4
F.D.	2.6 ± 0.7	10.5 ± 1.6	9.6 ± 1.9
L.D.	9.4 ± 1.4	11.4 ± 1.3	12.4 ± 1.9
No.	16.8 ± 3.4	17.0 ± 3.3	10.4 ± 2.2
Sh.	4.4 ± 0.9	5.2 ± 1.1	2.7 ± 0.6

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.* Beh., see Table 2.1 for description of behaviours.

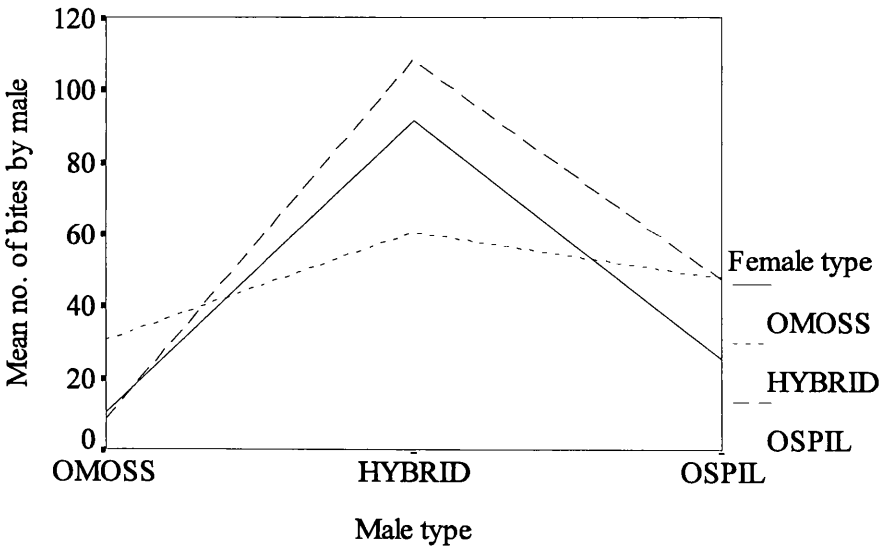


Figure 2.9 Mean number of bites performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid males in front of each type of female during the 30 minute Open-choice experiments. See Table I.9 (Appendix I) for standard error of means.

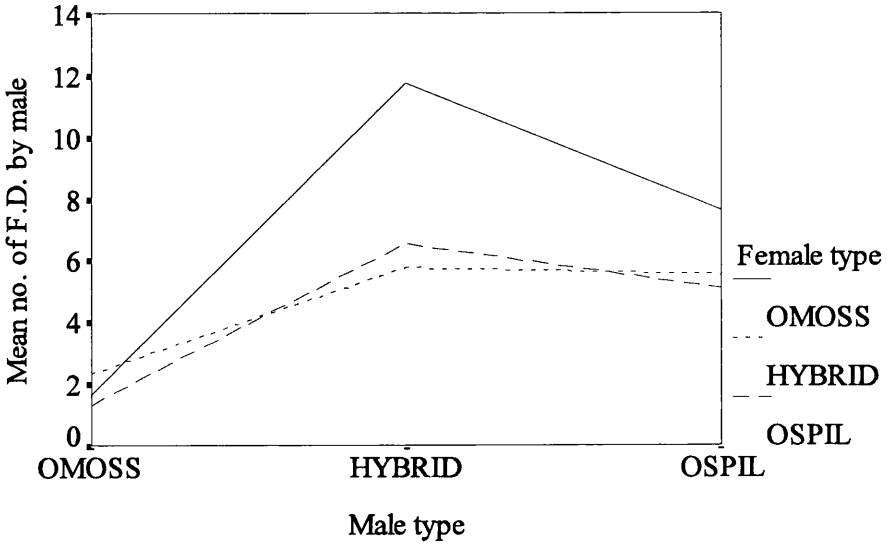


Figure 2.10 Mean number of frontal displays performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid males in front of each type of female, during the 15 minute 'Forced-choice' experiments. See Table I.7 (Appendix I) for standard error of means.

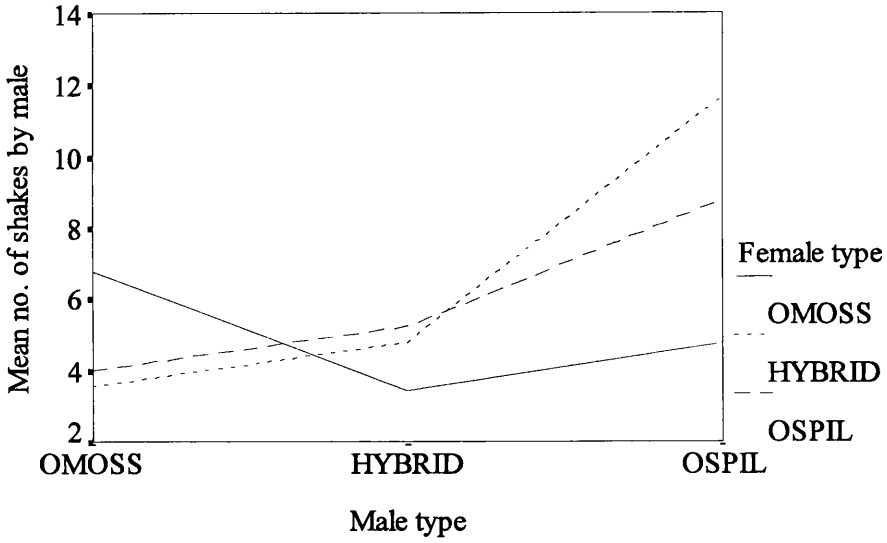


Figure 2.11 Mean number of shakes performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid males in front of each type of female, during the 15 minute Forced-choice experiments. See Table I.7 (Appendix I) for standard error of means.

B. The effect of male and female type on male behaviour - Predicting mate preference

In accordance with the design of Open-choice experiments, results are presented as the differences observed between male types in their behaviour to individual female types. In Open-choice experiments, where females were presented with two different male types, the behaviour of each male to the female differed significantly in 23 of 27 tests. As with female behaviour (Table 2.16), significant differences were found in at least two of the three replicate tests for all

combinations of fish, except in tests where hybrid females were given the choice of pure-bred males (Table 2.21). The frequency of shaking during Forced-choice experiments, was the only case in which a statistically significant interaction between female and male type was observed (Table 2.17). Both *O. mossambicus* and *O. spilurus* males performed more shakes, than heterospecific and hybrid males, in front of conspecific females (Figure 2.11). This trend was observed across most behaviours. *O. spilurus* males performed more shakes than hybrid males to *O. mossambicus* and hybrid females. Whereas, *O. mossambicus* males performed less shakes than hybrid males to heterospecific and hybrid females. *O. spilurus* males performed more shakes than *O. mossambicus* males to hybrid females (Figure 2.11 and Table I.7). The number of displays performed to hybrid

Table 2.21 Mean (± SE) number of displays performed by males, for six behaviours recorded during Open-choice experiments in tests where the two males presented to the female were of different types (three replicates of each test) and results of R X C Tests of Independence using *G*-test* (Means for tests where two males were the same, in Table I.8 Appendix I).

Female	Beh.	Male types presented to female					
		OMOSS & OSPIL		OMOSS & HYBRID		OSPIL & HYBRID	
OMOSS	Bi.	10.0 ± 6.4	21.0 ± 14.8	4.7 ± 4.7	126.7 ± 66.9	33.0 ± 16.1	126.7 ± 76.1
	Di.	15.3 ± 8.2	0.0 ± 0.0	22.3 ± 14.1	5.3 ± 1.2	9.0 ± 4.3	47.0 ± 36.5
	F.D.	7.3 ± 2.6	5.0 ± 2.5	1.0 ± 1.0	6.7 ± 3.7	11.3 ± 6.4	9.0 ± 1.0
	L.D.	16.7 ± 4.4	5.0 ± 1.1	14.7 ± 1.7	14.0 ± 6.6	15.0 ± 4.6	16.0 ± 1.1
	No.	7.0 ± 7.0	1.3 ± 1.1	26.3 ± 7.3	8.3 ± 2.6	17.3 ± 13.8	3.7 ± 1.8
	Sh.	8.0 ± 3.0	3.7 ± 2.7	6.7 ± 2.2	4.0 ± 3.0	4.0 ± 4.0	0.7 ± 0.7
Significant G-tests		(3) 13.94<*G*<24.18		(3) 31.62<*G*<219.27		(3) 27.91 <*G*<66.28	
OSPIL	Bi.	0.0 ± 0.0	50.7 ± 32.2	6.7 ± 5.2	126.3 ± 32.5	71.0 ± 36.1	64.3 ± 29.2
	Di.	8.3 ± 6.9	4.3 ± 2.3	4.7 ± 1.2	27.0 ± 11.3	9.3 ± 6.6	9.0 ± 7.1
	F.D.	0.0 ± 0.0	12.0 ± 6.8	2.7 ± 0.3	14.7 ± 5.7	10.3 ± 4.6	1.3 ± 1.3
	L.D.	6.0 ± 2.5	17.3 ± 2.8	12.0 ± 5.2	21.0 ± 9.1	16.7 ± 6.2	5.7 ± 3.2
	No.	5.7 ± 3.0	14.0 ± 6.5	12.7 ± 3.9	10.0 ± 1.1	15.7 ± 3.7	3.3 ± 0.3
	Sh.	2.0 ± 1.5	9.0 ± 3.2	9.7 ± 5.9	6.0 ± 2.3	2.7 ± 0.9	0.3 ± 0.3
Significant G-tests		(2) 15.15<*G*<111.07		(3) 24.57<*G*<89.07		(3) 51.00 <*G*<244.52	
HYBRID	Bi.	4.3 ± 1.8	2.0 ± 1.5	8.3 ± 4.3	5.0 ± 1.1	43.4 ± 25.7	70.0 ± 61.1
	Di.	11.7 ± 11.2	4.0 ± 2.6	18.3 ± 9.1	59.3 ± 9.4	3.3 ± 1.7	15.0 ± 8.1
	F.D.	3.7 ± 2.3	0.7 ± 0.7	1.0 ± 0.6	18.7 ± 9.2	9.0 ± 3.6	3.0 ± 0.6
	L.D.	5.0 ± 1.5	6.0 ± 4.2	7.7 ± 3.5	11.3 ± 6.4	6.7 ± 3.3	13.0 ± 5.2
	No.	4.3 ± 4.3	9.3 ± 7.0	0.7 ± 0.7	1.0 ± 1.0	25.0 ± 1.5	20.0 ± 9.5
	Sh.	4.0 ± 1.5	6.0 ± 5.0	5.3 ± 2.8	1.7 ± 0.3	2.3 ± 1.4	1.7 ± 1.2
Significant G-tests		(1) *G* = 32.28		(2) 16.47<*G*<57.83		(3) 30.93 <*G*<91.04	

OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.* Beh., see Table 2.1 for description of behaviours.
**Significant G-tests* - Number of tests out of three, where the number of male displays performed to the female (across all behaviours) are significantly different between paired males (in parenthesis), and all significant *G* statistics where *df* = 5, and when *G*= 11.07, *P* = 0.05; when *G*= 15.09, *P* = 0.01; and when *G*= 20.52, *P* = 0.001.

females, and by hybrid males, often varied between experiments and behaviours.

The following trends were observed in the behaviour of different male types to female types when the results, from Forced-choice tests (Table I.7, Appendix I), across all Open-choice tests (Table I.9, Appendix I) and from comparisons between paired males in Open-choice tests (Table 2.21), were compiled:

(1) *Pure-bred males to pure-bred females* - Across all experiments, *O. spilurus* males performed more displays than *O. mossambicus* males to *O. spilurus* females. *O. mossambicus* males generally performed more displays than *O. spilurus* males to *O. mossambicus* females (except biting). These results reflect female spawning preference.

2) Pure-bred and hybrid males to conspecific females - In Forced-choice experiments (Table I.7), and across all Open-choice experiments (Table I.9), *O. spilurus* males generally performed more displays than hybrid males to *O. spilurus* females (except biting and digging). However, when the mean number of displays given by paired males are compared, hybrid males displayed more than *O. spilurus* males to *O. spilurus* females (except shaking) (Table 2.21). Across all experiments, *O. mossambicus* males generally performed more displays than hybrid males to *O. mossambicus* females (except two aggressive behaviours - biting and frontal displays). As with female behaviour, a high frequency of displays only predicts mate preference in *O. mossambicus*.

3) Pure-bred and hybrid males to heterospecific females - Across all experiments, hybrid males generally performed more displays than *O. mossambicus* males to *O. spilurus* females. The number of displays performed by *O. spilurus* and hybrid males to *O. mossambicus* females varied across behaviours and experiments. In general, hybrid males performed more bites and digs, and *O. spilurus* males performed more shakes.

4) All males to hybrid females - In Forced-choice experiments, for all behaviours, hybrid males performed more displays than pure-bred males to hybrid females. In Open-choice experiments, hybrid males generally performed more displays than pure-bred males to hybrid females (except nodding and shaking). Across all experiments, in a comparison of the behaviour of pure-bred males to hybrid females, *O. spilurus* males performed more nods and shakes and *O. mossambicus* males performed more bites and digs. It is not possible to predict mate preference from the behaviour of males to hybrid females.

C. Interactions between the two males presented to the female

In Forced-choice experiments, nodding was the only behaviour in which the number of

displays increased when the two males consecutively presented to the female were of the same type (Table I.10, Appendix I). Across all behaviours, a significant interaction was observed between the two male types present in Open-choice tests (Table 2.18). For some behaviours, the number of displays increased when the two males presented to the female were of the same type. For example, each type of male performed more nods when the other male present (O.m.p) was of the same type (Figure 2.12, and Table I.11 Appendix I.). Pure-bred males performed the least number of nods when the O.m.p was a heterospecific, whereas hybrid males performed a similar number of nods when the O.m.p was *O. spilurus* or *O. mossambicus* (Figure 2.12). For other behaviours, the number of displays increased only when certain types of male were together. For example, the number of frontal displays increased when both males were *O. spilurus,* although the number of frontal displays performed by *O. mossambicus* males were similar (and infrequent) with all types of other male present. Hybrid males performed most frontal displays when the O.m.p was *O. mossambicus* (Figure I.2). The number of bites performed increased for both pure species when the other male present was a conspecific. Hybrid males performed far more bites than other males, and irrespective of the other male present (Figure I.3 and Table I.11, Appendix I).

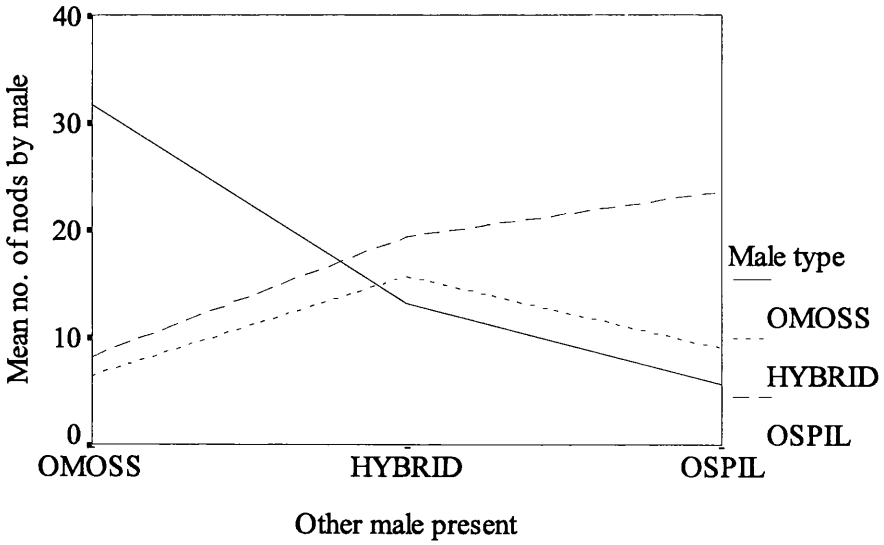


Figure 2.12 Mean number of nods performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid males in front of all females, with each type of other male present, during the 30 minute Open-choice experiments. See Table I.11 for standard error of means.

D. Interactions between the female and other male present

The only significant interaction between female type and the type of O.m.p occurred for the number of frontal displays performed by males (Table 2.18). More frontal displays were performed when the female and O.m.p were heterospecifics, and least when they were conspecifics.

The number of displays also decreased when the other male presented to a hybrid female was a hybrid male (Figure 2.13, and Table I.12, Appendix I). A similar pattern was observed for the number of lateral displays performed by males (Table I.12). In pure species, the decrease in the number of displays when the female and O.m.p are of the same type, is probably attributable to the reduced amount of time that females spent in front of males when the O.m.p was a conspecific.

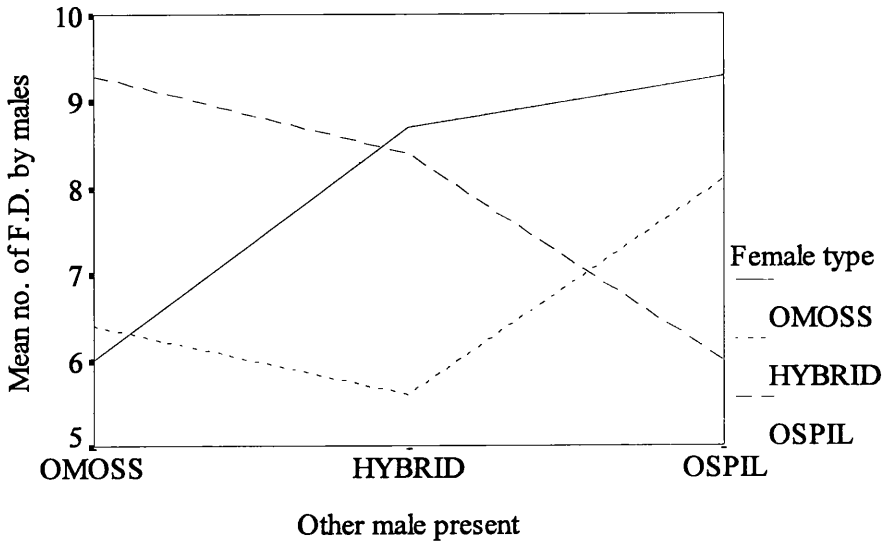


Figure 2.13 Mean number of frontal displays performed by all males in front of *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid females, with each type of other male present, during 30 minute Open-choice experiments. See Table I.12 (Appendix I) for standard error of means.

2.4 DISCUSSION

2.4.1 Mate choice in pure-bred and hybrid females

2.4.1.1 Mate choice in pure-bred females

When given a choice of pure-bred males, in mate choice tests with water contact, females always choose to spawn with a conspecific male (i.e. pure-bred fish mated assortatively). Pure-bred females also showed a preference for conspecific males, over heterospecific males, in their allocation of time between males. Females spent less time with a male if the other male present was a conspecific. In Forced-choice tests, despite the lack of visual contact with one of the males, time allocation was also affected by the type of other male present. When given a choice of conspecific and hybrid males, pure-bred females avoided mating with hybrid males and spent more time with conspecific males. *O. spilurus* females did not differ in spawning preference between hybrid and heterospecifics, but generally spent more time with hybrid males. *O. mossambicus* females showed a preference for hybrid males, over heterospecifics, in time allocation and spawning.

The only case in which a pure-bred female did not mate assortatively occurred in a mate-choice test where there was no water contact. In this test, the *O. spilurus* female spawned in front of an *O. mossambicus* male. Without water contact, males of the mouthbrooding cichlid *Haplochromis burtoni* responded in the same way to conspecific and *C. nigrofasciatum* females. When chemical information was also available, males preferred conspecific females (Crapon de Caprona 1982). Crapon de Caprona (1980) showed that *H. burtoni* is capable of distinguishing sex and stage of sexual readiness of conspecifics by chemical cues alone. Furthermore, *O. niloticus* males have been found to display preferentially near conspecific female pheromone (Falter & Dolisy 1989). Nevertheless, in mate choice tests based on visual cues alone, Falter & Charlier (1989) found that most *O. niloticus* and *O. mossambicus* females selected for conspecifics, and only one female of each pure-bred species choose a heterospecific mate (less than 9% of tests). Therefore, although chemical cues may play a role, they do not appear to be essential for interspecific mate recognition or for initiating courtship behaviour in *Oreochromis.* Furthermore, in this study in tests with water contact, both species spawned with a hybrid male on one occasion when they were given the choice of a conspecific and a hybrid male. It is possibly a lack of response and courtship behaviour by males that have lost interest in the female, which causes the female (in tests both with and without water contact) to not always choose the conspecific male (G. F. Turner *pers. comm.*). If females are not ready to spawn at the start of the test and show no sexual interest in the males, the males may lose interest and not respond to the female even when she is ripe. If neither male is courting (showing colours or displaying), the female will then spawn at random on either side of the tank. Otherwise, if both males are courting, the female can distinguish males on courtship behaviour and colouration alone.

The dominant role of courtship colouration in the interspecific female mate choice of cichlids has recently been illustrated by the study of Seehausen & van Alphen (1998). Mate choice tests were conducted on the two closely related Lake Victoria haplochromine cichlids (*Haplochromis nyererei* complex) which differ primarily in male colouration. Tests were carried out under monochromatic light, which masks interspecific difference in colouration, and under white light. Females of both species exhibited species-assortative mate choice when colour differences were visible, but chose non-assortatively when colour differences were masked by light conditions (Seehausen & van Alphen 1998). Reduced visibility, due to increased sedimentation, is thought to be responsible for recent cases of hybridization between haplochromine species in Lake Victoria (Seehausen *et al.* 1997). Similarly, poor visibility in richly fertilized farm ponds,

may prevent assortative interspecific mate choice in species of *Oreochromis* in aquaculture.

There are other factors, not studied in these experiments, which may contribute to interspecific mate recognition in *Oreochromis*, for example, secondary sexual structures such as the elaborate genital papillae of the *Oreochromis (Nyasalapia)*, and species-specific differences in the form of spawning pits (Fryer & Iles 1972; Turner & Robinson *unpubl.*). Nevertheless, where *Oreochromis* species have been introduced or where species are together in aquaculture, chemical and visual information do not prevent hybridization. Strong interspecific differences in breeding colouration have been ineffective in preventing hybridization, and isolation between species often appears to be purely geographical. For example, the naturally allopatric species *O. spilurus niger* and *O. leucostictus* differ greatly in colouration, but rapidly hybridized when introduced into Lake Naivasha (Elder *et al.* 1971). Elder *et al.* (1971) suggested that the value of specific breeding colouration and behaviour, as an isolating mechanism, appear to be relative to conditions under which it arose. In cichlid species, premating isolating mechanisms that evolved in native sympatric populations appear to be more effective than larger difference between allopatric populations (Elder *et al.* 1971; Stauffer *et al.* 1996). Nevertheless, in this study, the allopatric species *O. spilurus* and *O. mossambicus* did mate assortatively. Pure-bred females only spawned with heterospecific males when they had no other choice; a situation that may arise when one species is rare. As well as poor visibility, a rarity of conspecific mates is also a major factor in encouraging hybridization. Where species of *Oreochromis* have been introduced into the natural range of another species, it is highly likely that the population size of the introduced species is significantly smaller than that of the native (or established) species.

In situations where there are no conspecific mates available, female *Oreochromis* species will spawn with the next 'best' choice. Optimal choice behaviour models are often used to explain decision making in many aspects of animal behaviour, such as foraging and sexual selection (McFarland 1985; Bakker & Milinski 1991; Krebs & Davies 1991). When considering sexual selection, females 'sample' many conspecifics and choose the 'best' one. As the point to which the female has to spawn grows nearer, with restricted choice the female will choose progressively less attractive males. The female is eventually forced to accept males with few of the traits she is selecting for (a 'decaying threshold strategy'). Sexually selected traits include brightness, size, and aggressiveness (e.g. Barlow 1986; Bakker & Milinski 1991; Nelson 1995). In this study, the less favourable mates are hybrid or heterospecific males. This strategy has also been observed when naturally sympatric *Pseudotropheus* species from Lake Malawi are given a choice of hybrid,

conspecific and heterospecific mates (G. F. Turner *pers. comm.*).

Assortative mating in *O. spilurus* and *O. mossambicus* provides support for them as 'true' species. Since *O. spilurus* and *O. mossambicus* are allopatric and there cannot be the opportunity for direct selection for reproductive isolation (i.e. reinforcement), assortative mating must result from some consequence of intraspecific mate choice. Differences in mate preference (i.e. traits which are selected for by females) may occur between allopatric and sympatric populations, through sexual selection. Characters subject to high variance in female choice, such as the behaviour and appearance of a male during courtship, could change quickly due to local differences in female choice (Lande 1981, 1982). Indeed, mate choice is likely to have played a large role in the divergence of breeding colouration and speciation between African cichlids (Dominey 1984; McKaye *et al.*1993; Turner & Burrows 1995; Seehausen *et al.* 1997; van Oppen *et al.* 1998).

2.4.1.2 Mate choice in hybrid females

Hybrid females did not mate assortatively (i.e. they did not always prefer hybrid males), preferring to spawn with *O. mossambicus* males over both *O. spilurus* and hybrid males. Hybrid females rarely spawned with *O. spilurus* males, preferring hybrid males. Hybrid females generally spent more time in front of the hybrid male during Open-choice tests, and spent longer with *O. mossambicus* than with *O. spilurus* males. Hybrid females also spent more time in front of *O. mossambicus* males in Forced-choice tests. Falter & Charlier (1989) found that hybrid females failed to show any preference between *O. niloticus* and *O. mossambicus* males, in time allocation and spawning partner. However, hybrid males have been found to be more attracted by the pheromone of *O. niloticus* females over that of *O. mossambicus* females (Falter & Dolisy 1989). The present study provides no explanation as to why hybrid females show such a preference for *O. mossambicus* males and a 'dislike' for *O. spilurus* males. The mating preference of pure-bred females and hybrid females suggests that isolating mechanisms are most efficient in *O. spilurus*, because females showed no preference between hybrids and heterospecifics, and spawning was less likely between *O. spilurus* males and heterospecific or hybrid females. The design of this study, where females choose between males, best reflects the situation in nature. Females are thought to be the more 'choosy' sex because males have higher reproductive rates than females and have less to lose in terms of energy and time. However, based on these results it may be valuable to see if *O. spilurus* males show any preference between females in interspecific encounters.

In initial mate choice tests, hybrid females spawned significantly more frequently than

pure-bred females, although in additional mate choice tests, hybrid females generally took the same time to spawn as pure-bred females. Falter & Charlier (1989) found that hybrid females spawned more frequently than pure-bred females and, as in this study, also spawned in isolation. Laboratory observations have also shown that hybrid females start spawning earlier in life and lay eggs at a regular interval. The increased fecundity of hybrid females could be considered an effect of heterosis (Falter & Charlier 1989), and may accentuate the problems of precocious maturation often seen in the aquaculture of *Oreochromis*. Furthermore, the enhanced reproductive potential of hybrids allows them to colonize waters quickly after introductions, often becoming a pest and out-competing the endemic species (e.g. feral populations of *Oreochromis* hybrids in Australia; Mather & Arthington 1991).

2.4.1.3 Consequences of hybrid mate choice in the wild

Since hybrid females do not show a preference for hybrid males, over both the parental species, it is unlikely that a hybrid population could be maintained as a separate species in co-existence with parental species. Nevertheless, the maintenance of hybrid populations would depend on other factors such as their competitive ability relative to the parental species, their fertility and, if hybrids are not better competitors, on the availability of an ecological niche (Crapon de Caprona 1986a). However, the parental species cannot be maintained either if they continue to hybridize. In this study, the hybrid female showed a preference for one of the parental species, but it is difficult to determine how this may affect the fate of the parental species. At the initial stage of hybridization, most post F_1 progeny are likely to represent the progeny of hybrids back-crossing with the parental species, due to the greater number of parental species (Verspoor & Hammer 1991). In cases where species of *Oreochromis* have been introduced into the range of an established species (not necessarily an endemic species), and hybridization has occurred, it has often resulted in the elimination of one of the species followed by the disappearance of the hybrids (Moreau 1983; Ogutu-Ohwayo & Hecky 1991). In this study, based on the mate preference of the parental species and hybrids, one could predict that the genetic integrity of *O. spilurus* is more likely to remain intact, and *O. mossambicus* more likely to be modified. However, the feasibility of such predictions cannot be substantiated without knowledge of the competitive abilities of the two species in the habitat to which they have been introduced. Whatever the outcome, it is highly likely that the genetic composition of the parental species will be permanently altered following an incidence of hybridization. For example, the populations of *O. niloticus* in Lake Itasy are

characterized by poor growth rate (Daget & Moreau 1981). Genetic introgression in natural trout populations, through the introduction of farmed strains or different species, has commonly lead to a decline in the native population (Leary *et al.* 1993; Campton & Johnston 1985).

2.4.2 Differences in behaviour of *O. spilurus, O. mossambicus* and hybrid fish

2.4.2.1 Species-specific differences in behaviour

No differences were observed in the form of behaviours between *O. mossambicus* and *O. spilurus*, although it was difficult to make direct comparisons between fish and identify such differences. Furthermore, to an inexperienced observer, differences in the form of behaviours between species may not be apparent. The intensity and duration of displays may also vary between species, although no variation was noted and such differences would be difficult to quantify. More detailed and accurate comparisons could be made with the use of video recordings. Incidently, it was presumed that the experimental fish were pure-bred, and that their behaviour (form and frequency of displays) was, therefore, representative of the species. Some differences in the forms of behaviours have been observed between species of *Oreochromis,* for example *O. mossambicus* was found to court with the dorsal fin depressed, while *O. niloticus* kept the dorsal raised (Baerends & Blokzijl 1963).

In this study, species-specific differences were found in the frequency of display of some behaviours. For example, *O. mossambicus* females performed fewer frontal displays, and more nods, than *O. spilurus* females. *O. mossambicus* males performed fewer frontal displays and bites than *O. spilurus* males. The observed differences in the frequency of behaviours suggest that *O. mossambicus* may be less aggressive than *O. spilurus*. *O. mossambicus* has also been found to be less aggressive than *O. niloticus* (Baerends & Blokzijl 1963; Falter & Charlier 1989). Baylis (1976b) found no evidence of species-specific behaviour between *Cichlasoma citrinellum* and *C. zaliosum,* but substantial differences in the frequency of courtship behaviours was shown. The differences were explained by the lower threshold of aggression in *C. zaliosum* and the lower threshold of sexual behaviour in *C. citrinellum.* Differentiation in colour between these species is minor and there is no habitat or temporal isolation. Therefore, isolation depends on an inappropriate response by a heterospecific. Courting is also dependent on the correct response of females in *O. mossambicus* (Neil 1964).

It has been suggested that differences in colouration play a secondary role to differences in behaviour, and spawning is less likely to occur when the female belongs to the more aggressive species (Falter & Charlier 1989; Falter & Dufayt 1991). However, the current study provides no

support for this theory. *O. spilurus* was found to be the most aggressive and *O. mossambicus* the least aggressive. If aggression is important, it would have been expected, for example, that spawnings between *O. spilurus* females and *O. mossambicus* males would have been less frequent than between *O. spilurus* females and hybrid males. However, *O. spilurus* females showed no preference between hybrid and non-conspecific males. Furthermore, females are never aggressive while courting under natural conditions (G. F. Turner *pers. comm.*).

Differences in behaviour between species, or populations, may occur through adaptations to a particular habitat (Hinde 1959). The divergence of courtship displays in three *Haplochromis* species has been attributed to selection for responsiveness to different types of environmental stimuli in each species: to prey; to disturbance; and to territorial intruders (Carlstead 1982). Selection for optimal levels of aggression or timidity to different environmental stimuli, may explain the different levels of aggression between *Oreochromis* species. However, species of *Oreochromis* generally have very similar environmental requirements and stimuli, and there is no evidence to support this explanation. As previously suggested (Section 2.4.1.1), species-specific differences in behaviour (aggressiveness, frequency of display etc) may be due to sexual selection resulting in local differences in female preference.

2.4.2.2 Characteristics of hybrid behaviour

The behaviour of hybrid fish was largely characterised with respect to pure-bred fish, by the greater frequency of biting and digging displays. These results are largely in agreement with those of Falter & Charlier (1989), who proposed that hybrid females (*O. mossambicus* x *O. niloticus*) showed more aggressively motivated behaviour (biting, lateral displays and digging) than the parental species. Digging may be interpreted as an aggressive behaviour in the establishment or territories, or as sexual behaviour when both sexes are preparing to spawn. Indeed, certain actions take on different meanings according to the context used (Fryer & Iles 1972). The motivational reasons for fish biting the partition separating them from the opposite sex, is also unclear. Biting is generally perceived as an aggressive behaviour between fish during fighting, where physical contact is made (Neil 1964). The biting of plastic partitions is a behaviour induced by the restrictions of the aquarium environment. This behaviour may be agonistic or it may be a reflection of the fishes efforts to 'get closer' to the opposite sex. However, it seems more plausible that both digging and biting are aggressively motivated behaviours due to the high frequency with which they are performed and the general low frequencies of sexually motivated behaviours (such

as quivering and skimming).

Of the other behaviours observed, the frequency of shaking behaviour in hybrid fish, of both sexes, was more similar to *O. mossambicus* than *O. spilurus*. The frequency of nodding by hybrid females was more similar to *O. spilurus*, whereas the frequency of nodding in hybrid males was less than either of the pure species. Overall, it does not appear that the behaviour of hybrids was intermediate to that of the parental species. On the basis of aggressiveness, hybrid fish resembled their maternal parent (*O. spilurus*) more in this respect. In the discriminant analysis of social behaviour of the brook charr (*Salvelinus fontinalis*) and lake charr (*S. namaycush*), Ferguson & Noakes (1983) found that hybrids and backcrosses showed a general tendency towards intermediacy, but with a closer resemblance to the maternal parent. The courtship behaviour of hybrids, between the swordtail (*Xiphophorus helleri*) and platyfish (*X. maculatus*), also has a generally tendency towards intermediacy (Clark *et al.* 1954). Most behavioural traits appear to be associated with differences in a number of loci, and therefore species-specific differences in behaviour are thought to have a polygenic basis of inheritance (Ferguson & Noakes 1983; Danzmann *et al.* 1993). This could also explain the large variation observed in the behaviour of hybrids. Nevertheless, it is unlikely that the hybrids used here are fully representative of hybrids if *O. mossambicus* and *O. spilurus* were to come into contact in nature. All hybrids were from one brood and are therefore only a small sample of the possible combination of genes in the parental species. Furthermore, experiments were not conducted using hybrids of the reciprocal cross (*O. spilurus* female x *O. mossambicus* male). The behaviour and appearance of these hybrids may differ from the fish used in this study, particularly in sex-linked traits.

2.4.3 Factors affecting the frequency of displays

Factors affecting the frequency of displays were complex. In females, the frequency of displays was dependent on all three types of fish present. For the behaviour of both sexes, interactions between male and female types were not generally statistically significant (probably due to a lack of replicates and the high variability of display rate). However, in comparisons on behavioural data from individual Open-choice tests, in over 66% of tests the female performed a significantly different number of displays to the two males, and in over 85% of tests the two males performed a significantly different number of displays to the female. Display rates to different fish, by males and females, are discussed in relation to spawning preference. Behaviour was sometimes affected by the type of female and other male present. For both sexes, the number of displays

performed was greatly affected by the two types of male present. The interaction between male types is discussed in relation to experimental design, the role of chemical cues and its significance in the natural environment.

2.4.3.1 Interactions between the male and female - Predicting mate preference

For pure-bred fish, of both sexes, the number of displays performed by a conspecific fish were usually greater than the number of displays performed by a heterospecific. Similar results were obtained from Forced-choice and Open-choice tests. Displays for both sexual and agonistic behaviours were often greater in front of conspecific fish, so these results do not necessarily indicate that the fish showed a preference for conspecifics. Nevertheless, these results suggest that fish showed a greater 'interest' in conspecifics, and displays from conspecifics induced a greater response than displays from heterospecifics. Furthermore, behaviours for which the number of displays in front of the preferred mate were not greater, can often be explained by the 'meaning' of the behaviour. For example, *O. mossambicus* males generally displayed more than *O. spilurus* males, in front of *O. mossambicus* females, except for biting. In this case, the lower frequency of an aggressive behaviour, may also be interpreted as a preference for *O. mossambicus* females. Falter & Charlier (1989) found similar results, for example, *O. mossambicus* females performed some sexual behaviours at a higher rate than *O. niloticus* females, in front of *O. mossambicus* males.

The interpretation of display rate to mate preference when hybrid fish are involved, is more complex than the pattern observed for pure-bred fish alone. *O. spilurus* females displayed more to hybrid males than to conspecific males, except for nodding and shaking behaviour (both sexual behaviours). There was no consistent pattern in the behaviour of *O. spilurus* and hybrid males to *O. spilurus* females. Across all experiments, hybrid fish often showed a greater frequency of display for some behaviours (Section 2.4.2.2). The greater rate of display to hybrid males, by all females, may be due to a feedback response by females towards the behaviour of hybrids. There was no consistent pattern in the behaviour of *O. mossambicus* females to hybrid and *O. mossambicus* males. In front of *O. mossambicus* females, *O. mossambicus* males generally performed more displays than hybrid males, except for biting and frontal display behaviour. When the number of displays between the female and pair of males from individual Open-choice test were compared, the results largely predicted the mate preference of pure-bred females for conspecific males over hybrid males.

Pure-bred females performed more displays in front of hybrid males than in front of heterospecific males, although *O. spilurus* females showed no preference difference between hybrid and heterospecific males in spawning. *O. spilurus* males also displayed less than hybrid males, in front of *O. spilurus* females. The response of hybrid and *O. spilurus* males to the *O. mossambicus* females did not follow the previous patterns of behaviour or predict mate preference; hybrid males performed more aggressive behaviours (bites and digs) and *O. spilurus* males displayed more shakes. In general, there was no consistent pattern to the behaviour of males to hybrid females, or of hybrid females to males. Falter & Charlier (1989) also found that hybrid females showed no difference in the frequency of behaviours between *O. mossambicus* and *O. niloticus* males.

2.4.3.2 Interactions between males

The number of displays performed were often higher when the female was presented with males of the same type. For females during Open-choice tests, all behaviours except digging and shaking, were performed at a higher rate in at least one of the tests where the two males presented were of the same type. The display rate may have increased because females were receiving the same type of 'signal' from both males, thereby resulting in an increased response from the female. Even though females did not have visual access to one of the males during Forced-choice tests, the results were similar to those of the Open-choice test. If it is presumed that pheromones were freely distributing throughout the tank (from all fish), the similar results obtained from both tests illustrates the important role that chemical cues play in behaviour and communication. The presence of the same type of pheromone may 'excite' the female more than the presence of two different types of pheromone. The stronger relationship between male types and display rate in Open-choice tests, suggests that both visual and chemical cues are contributing factors to this response. The large variability in the number of displays performed by hybrids, and possibly in pheromone composition, may mean that both males are not giving out 'signals' as similar as those from two pure-bred males. This may explain why the number of displays did not always increase when both males were hybrids.

The relationship between the types of male present and the number of displays performed by males was not as consistent as that observed in female behaviours. For example, the number of frontal displays was higher when both males were *O. spilurus*, although the number performed by *O. mossambicus* males was similar with all other types of male present. There are several

explanations for this observation. Males were situated at either end of the tank, with several plastic (unsealed) partitions in between. This may have restricted the movement of pheromone from one end of the tank to the other, so that either fish could not always 'detect' which other male was present, although this is unlikely. Males had no visual access to each other, so it is unlikely that they could directly influence each other through displays. Therefore, the increase in display rate may have been partly due to the response of the female. The increase in displays by females, when the two males present were of the same type, may have subsequently also excited the males to a greater extent. If the response of the males was dependent on the female, this may also explain why the number of displays was not as consistently affected as those of the female. In Forced-choice tests, one of the males did not have visual access to the female. This may explain why the display rate of very few behaviours was affect by the type of males present.

To my knowledge, the interaction between the rate of behaviour and the type of fish present has not been reported before. It is difficult to determine the significance of this interaction in nature. The increase in responsiveness of the female may alert males to the fact that female has previously visited a conspecific male. However, in nature the spawning territories of males are often in close proximity and territorial males are usually in sight of neighbouring fish (Bruton & Boltt 1975) and therefore probably aware of visiting females. The *Oreochromis* species of Lake Malawi tend to nest well apart from conspecifics and interactions between neighbouring males are extremely rare (Turner & Robinson *unpubl.*). Furthermore, *O. mossambicus* males can be solitary as well as occurring in 'leks' (Nelson 1995). In these situations, the response of a female may be of more value to the male in assessing whether competing males are present. However, the frequency of mainly aggressive, rather than sexual, behaviours was increased in females when the two males were of the same type. Furthermore, the frequency of displays often increased when the two males were of a different type to the female. Females would not normally be expected to interact with heterospecific males in natural conditions. Therefore, it seems more plausible that the interaction is largely an artefact of the experimental design and laboratory conditions, where the female responds more because she is getting the same 'signals' from both males.

2.4.4 Experimental problems

2.4.4.1 Affects of captivity on behaviour and restrictions in the aquarium environment

The inherent problem of studying behaviour in captivity is the effect of the restricted environment. The possible influences of the observer, in cases where recording equipment is not

used, must also be considered. Under natural conditions the male's territories are considerably larger (often up to 3-5 m in radius; Baerends & Baerends-von Roon 1950) than the dimensions of a typical tank. In this study, the experimental tank was designed with central partitions, to compensate for the restricted space, although a larger tank would have been preferred. The behaviour of cichlids in aquaria has often been found to differ from that in the natural habitat (Rothbard 1979; Schwanck 1989). Digging activity, a behaviour particularly common in this study, has often been found to far exceed that in the wild. Heiligenberg (1965) suggested that this may be a response to the lack of predators or motivational conflict, since digging is considered to share motivational background with attack readiness. In this study, the construction of a spawning pit near one particular male by the female (that had spawned) was used to indicate the choice of mate. However, in the natural environment, females of *Oreochromis* species do not dig spawning pits. The influence of captivity on biting, possibly an aggressively motivated behaviour, has previously been discussed (section 2.4.1.2). Robinson & Turner (1990) found that the cichlid *Hemitilapia oxyrhynchus* was generally more aggressive in captivity compared to observations made in the field, and adopted colour patterns and social behaviour rarely, if ever, used in the natural environment. The authors suggested that cichlid fish are behaviourally flexible and may retain a complex suite of social and behavioural characteristics which are never expressed in nature.

2.4.4.2 Problems in experimental design and further studies

There were a number of problems with the design, analysis and implementation of the experiments that may have influenced the results. Firstly, only the number of displays was considered and some preference may have been shown by intensity and duration of displays. Preference may have also been expressed in other ways, such as in the number of eggs spawned (see Nelson 1995). It is also possible that the same behaviour may have a different meaning depending on the context with which it is displayed (such as agonistic or sexual). Furthermore, the analysis was based on the total number of displays in the experiment, and it may have been more accurate to consider the number of displays per unit of time. However, such analysis would have been complicated by the increasing rate over time with which some behaviours appear to be performed. Nevertheless, spawning in front of the chosen male was the definitive indication of mate preference by the female.

The statistical robustness of behavioural results are also of some concern. The small number of statistically significant differences, despite the apparent patterns in the results can be

attributed to the large variation in results, exacerbated by the small number of replicate tests. Furthermore, it would have been preferential to use data from independent tests in the same statistical analysis. Analysis based on data from independent tests produced very few significant results due to the lack of replicates. It would be valuable to repeat the experiments with a larger number of replicate tests, from independent experiments, and thereby determine if the current results are repeatable and representative.

Due to a restriction in the number of fish available, the majority had to be used in more than one experiment. This seemed to have no effect on the frequency of spawning because females were only used again once they were gravid. Nevertheless, prior mate choice experience of the female may have affected behaviour and mate choice in subsequent tests. There were no obvious differences, in the frequency of displays or the latency of spawning, between tests where females had been used and had not been used before. Similar problems apply to the repeated use of some males which could possibly gain experience and therefore have an advantage, although no one male was preferred consistently over another. On the other hand, with no physical contact with the females, the male may habituate to the sight of the female, which was in fact observed in the experiments with no water contact. In mate choice tests carried out by Falter & Charlier (1989), males were changed after several experiments because social isolation between tests reduced their territorial behaviour and therefore readiness to spawn. The repeated use of individuals is also undesirable since their behaviour may not be representative of the species.

Despite efforts to use only females which appeared to be in spawning condition, and at approximately the same level of ripeness, the time taken for some females to spawn during tests (up to 34 days) is of some concern. Similarly, the infrequent occurrence of skimming and quivering behaviour in both sexes may also suggest that fish were unreceptive. Skimming and quivering are both intense behaviours which typically occur close to spawning. The lack of physical contact possibly prevented the fish from reaching that stage of courtship since skimming was observed on one occasion after the removal of the partition. Furthermore, observations were made only on the behaviour of males and females during their first encounter. It may be more valuable to monitor behaviours in time blocks up to the act of spawning (as in a study mate choice within *O. mossambicus;* Nelson 1995), which may reveal more differences in the frequency of courtship displays between intraspecific and interspecific encounters. It would also be valuable to repeat the Forced-choice tests, where differences in the frequency of behaviours are easier to monitor than in tests where the female may have the choice not to interact with a heterospecific. In this study,

the Forced-choice experiments did not really have the desired effect because of the influence of the other male present. However, these experiments were valuable in illustrating the possible influence of chemical cues on behaviour and their importance in intraspecific mate recognition.

2.4.5 Conclusion

Pure-bred females mate assortatively, showing a preference for conspecific males, over heterospecific and hybrid males, in time allocation and spawning. When pure-bred females have no other choice, either due to an absence of conspecific males or lack of response by conspecific males, they will spawn with heterospecific and hybrid males. Hybrid females spawned more frequently in front of *O. mossambicus* males, and preferred hybrid males over *O. spilurus.* Time allocation did not always predict mate preference in hybrid fish. The behaviour of *Oreochromis* species and their hybrid was found to be a complex process influenced by a number of interacting factors. Differences were found in the frequency of displays between sexes, and between all types of fish of both sexes. The behaviour of hybrids was not intermediate to that of the parental species and resembled the maternal parent (*O. spilurus*) in terms of aggressiveness. Pure-bred fish behaved differently to conspecific and heterospecific fish, both sexes displaying more to conspecifics. Pure-bred fish did not always display more to their preferred mate when given a choice of hybrid and pure-bred fish, and the behaviour of hybrids did not generally predict mate preference. The frequency of male and female displays was also affected by the two types of male presented to the female. The interpretation of behavioural results, in relation to mate choice between pure-bred and hybrid fish in the wild, is often difficult and the influence of experimental conditions must be taken into account. A greater number of replicate results, from independent tests, would greatly improve the statistically robustness of the results and may clarify patterns of behaviour. Further experiments, in which the reciprocal cross of the hybrid is also used, would be more representative of the behaviour of hybrid fish.

2.5 SUMMARY

The findings of this study can be summarised as follows:

1) Females of the allopatric species *O. spilurus* and *O. mossambicus* mated assortatively. Pure-bred females preferred conspecific males over heterospecific and hybrid males, in time allocation and spawning. When pure-bred females have no other choice they will spawn with heterospecific and hybrid males.

2) Hybrid females showed a mate preference for *O. mossambicus* males over hybrid males, and preferred hybrid males over *O. spilurus.* Allocation of time between males did not always predict spawning preference.

3) In the study of aggressive and sexual behaviours during encounters between *O. spilurus, O. mossambicus* and their hybrid, differences were found in the frequency of displays between sexes, between species and between pure-bred and hybrid fish.

4) For some behaviours, hybrid fish performed more displays than the parental species. The behaviour of hybrids was often more variable than the pure-bred fish. Hybrids resembled the maternal parent (*O. spilurus*) in terms of aggressiveness.

5) Factors determining the frequency of behavioural displays were complex. The frequency of displays differed under different mate choice conditions, and were dependent on the type of male and female tested and on the two types of male presented to the female.

6) Pure-bred fish behaved differently to conspecific and heterospecific fish, both sexes displaying more to conspecifics. Pure-bred fish did not always display more to their preferred mate when given a choice of hybrid and pure-bred fish. Behaviour did not generally predict mate preference in hybrid fish.

7) Results were very similar between Forced-choice and Open-choice tests, probably because in both tests fish were in water contact. Forced-choice tests illustrate the influence of chemical cues in behaviour and communication.

8) It would be valuable to repeat tests with more replicates and with the reciprocal hybrid, and to study differences in the behaviour of fish up to the point of spawning.

CHAPTER 3
HYBRIDIZATION IN FARM PONDS, MALAWI: EVIDENCE FROM ALLOZYME ELECTROPHORESIS

3.1 INTRODUCTION

The tilapiine cichlids found in Lake Malawi include *Tilapia rendalli*, *Oreochromis (O.) shiranus*, and the 'chambo' species of the subgenus *Oreochromis (Nyasalapia)* (Trewavas 1983). These species, particularly chambo, are an important component of the fishery in Lake Malawi. Lake Malawi is the only large species-rich lake to contain a tilapia flock that may have evolved within the lake in the presence of competing haplochromines (Trewavas 1983; Turner & Robinson 1991). The *Oreochromis (Nyasalapia)* of Lake Malawi comprise a monophyletic flock that consists of three species: *O. karongae*, *O. lidole* and *O. squamipinnis* (Turner & Robinson 1991). These species exhibit several unique synapomorphies (Trewavas 1983; Turner *et al.* 1989b; Turner & Robinson 1991) and a very low level of genetic differentiation (Sodsuk *et al.* 1995). The difficulties in identifying the chambo species reliably, except as sexually mature adult males, have been a major constraint in their management. Due to overfishing, chambo are a presently declining component of the fishery in Lake Malawi (Turner 1995). As the population of Malawi continues to grow it is likely to become increasingly reliant on aquaculture (Brummett 1995).

Aquaculture in Malawi is still in its early stages and has suffered in the past from limited funding and research. More recently, the Malawi Government has developed an aquacultural policy to encourage fish farming as a means to supplement fish production from capture fisheries. In 1995 there was estimated to be over 2000 fish farmers (Maluwa *et al.* 1995). The species *O. karongae, T. rendalli* and *O. shiranus,* are commonly grown in monoculture, or in polyculture using the catfish *Clarias gariepinus* with either *O. shiranus* or *O. karongae* (Maluwa *et al.* 1995). *O. shiranus* has been distributed widely as a farmed species throughout Malawi and often stocked into small water bodies (Mattson & Kaunda 1997). However, since *O. shiranus* and *T. rendalli* exhibit poor growth due to precocious breeding behaviour, *O. karongae* has often been favoured due to its later maturation and thereby better growth performance (Maluwa & Dickson 1993). Many fish farms, particularly experimental ones, keep both *O. shiranus* and chambo species. There is anecdotal evidence (based on morphological characters) that the introduction of *O. karongae* into ponds containing *O. shiranus* has resulted in the formation of interspecific hybrids (A. Brooks and G. F. Turner; *pers. comm.*).

The unintentional production of hybrids, within farm ponds or via the release of fish into the wild, and the resulting deleterious implications for the conservation of their genetic resource, has commonly been documented in tilapia (see Chapter 1). Introgression may result in the loss of the unique qualities of the native species that were previously useful for fisheries and aquaculture. Mature lake communities often have high species diversity and marked endemism making them very susceptible to any changes, including fish introductions. The Malawi Government is concerned about the preservation of unique faunas and has a strong policy against species introductions. This has led the Fisheries Department to ban common carp culture in the Lake Malawi catchment. However, the widespread escape of tilapia hybrids from Malawian farm ponds into the wild could form a route for natural hybridization, which has thus far not been documented. The ecological requirements of the chambo species are very similar. A great overlap is observed between breeding sites, breeding season and morphology (including the colours of sexually mature males) and it is not certain which factors maintain reproductive isolation between chambo species (Turner & Robinson 1991; Turner *et al.* 1991a, b).

Allozyme electrophoresis, a method independent of the often unreliable morphological characters, has commonly been used to identify hybrids of tilapia (e.g. Taniguchi *et al.* 1985; Macaranas *et al.* 1986; De Silva & Ranasinghe 1989). Detecting hybridization and introgression by electrophoretic methods is relatively straightforward, especially, when the two parental species are fixed for different alleles at two or more loci. In these circumstances, F_1 hybrids will be heterozygous for the different parental alleles at all diagnostic loci. Later generation hybrids will express a broad mixture of recombinant types, including the two parental types (Campton 1987, 1990). Due to its ease, speed and relatively low cost, allozyme electrophoresis remains a popular technique for the detection of hybridization. Sodsuk *et al.* (1995), in an analyses of 43 enzyme loci, identified five loci with fixed alleles which could clearly separate *O. shiranus* from chambo. However, no fixed difference between *O. karongae*, *O. lidole* and *O. squamipinnis* were observed. There has been no previous genetic attempt to examine the status of chambo species grown in culture or to identify hybrids between *O. shiranus* and chambo.

The aims of this study, using allozyme electrophoresis analysis, were:

1) To identify diagnostic loci for discriminating the three chambo species and *O. shiranus*.

2) To investigate the genetic status of the *Oreochromis* species in wild and farm pond populations.

3) To provide molecular genetic evidence for the occurrence, or not, of hybrids between chambo and *O. shiranus* in farm pond populations. In turn, to determine the incidence of hybridization and

whether the hybrid individuals are F_1 or post-F_1 generation (i.e. establish if hybrids are fertile).

4) To examine the effects of stocking history (e.g. species used, density, sex ratios) and environmental conditions (e.g. water clarity, nesting area available *etc.*) on the incidence of hybridization in farm ponds.

5) To identify possible avenues for the escape of culture fish into the wild, and assess the evolutionary implications of any such escape.

Complementary studies were undertaken on the same samples using RAPD analysis (Chapter 4) and morphometric analysis (Chapter 5).

3.2 MATERIALS AND METHOD

3.2.1 Sampling

Samples were collected during a three week trip (19-11-95 to 9-12-95) to Malawi (Figure 1.1, Chapter 1), in late November to early December to coincide with the breeding seasons of the four species being sampled. The reliable identification of species, particularly the three chambo species, was possible only using large mature males in breeding colours. Characteristics of each 'type' species were used for identification (G. F. Turner *pers. comm.*, Table 3.1, Figures 3.1 - 3.4). The identification of the subspecies *O. shiranus shiranus* and *O. shiranus chilwa* was not assessed, and therefore both were collectively referred to as *O. shiranus*.

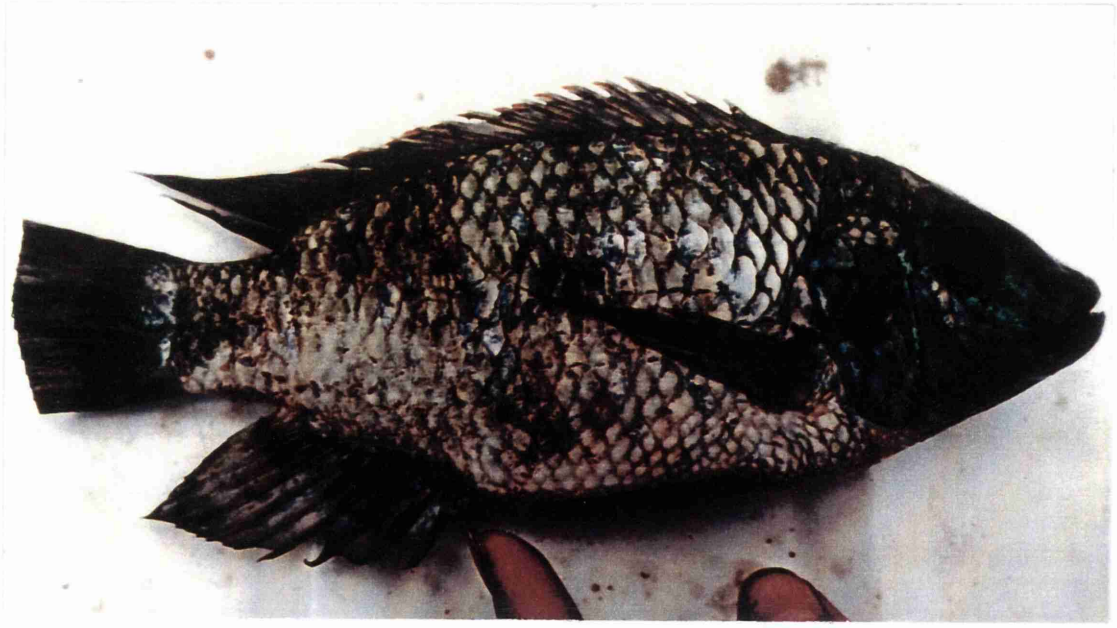


Figure 3.1 Male *Oreochromis (O.) shiranus* - note that the caudal fin has been clipped, removing most of the distinctive red margin (Photograph by Eric Roderick).

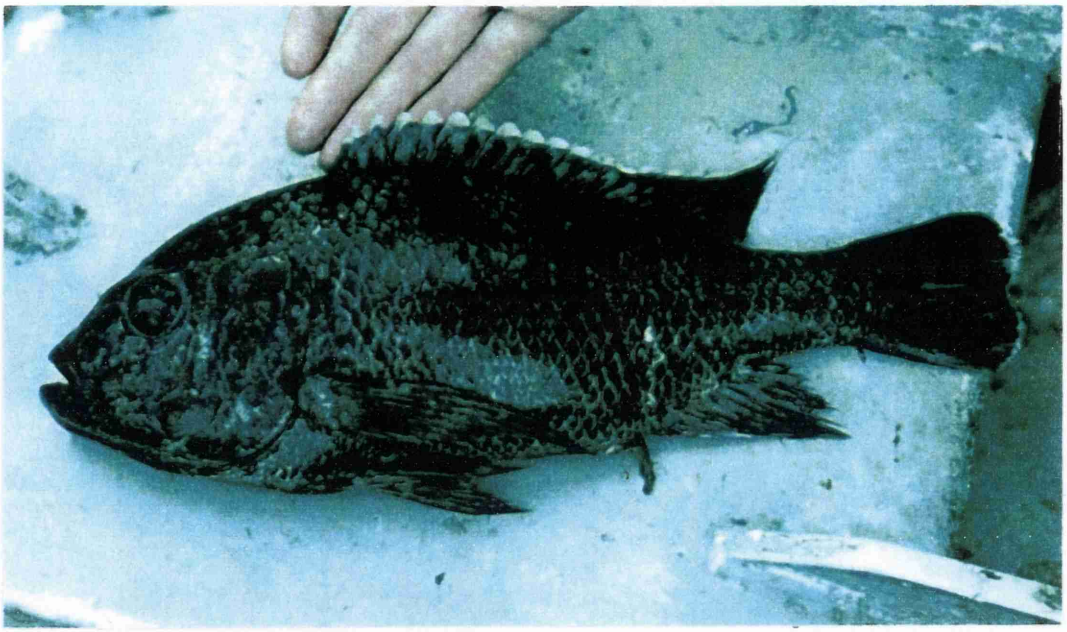


Figure 3.2 Sexually mature male *Oreochromis (Ny.) lidole* in full breeding colours. Note the genital tassel - a characteristic of male *Oreochromis (Nyasalapia)* (Photograph by Eric Roderick).

Figure 3.3 The distinctive face mask of male *Oreochromis (Ny.) squamipinnis*, varying in colour from almost white to bright turquoise, shown in comparison to the face of *Oreochromis (O.) shiranus* (far left) (Photograph by Eric Roderick).

Figure 3.4 The three *Oreochromis (Nyasalapia)* of Lake Malawi - *Oreochromis (Ny.) lidole* (top), *O. (Ny.) karongae* (middle) and *O.(Ny.) squamipinnis* (bottom) (Photograph by George Turner).

Table 3.1 Comparative morphological characters used for the identification of *O. shiranus* and chambo species, with details of characters specific to individual chambo species (*O. lidole, O. squamipinnis* & *O. karongae*). Abbreviations used for species are shown in parenthesis.

Character	*O. shiranus* (OSHI)	Chambo	*O. squamipinnis* (OSQU)	*O. karongae* (OKAR)	*O. lidole* (OLID)
Body colour	Golden yellow	Silvery grey		Coppery	Slaty grey
Pattern	Horizontal stripes	Vertical bars			
Ripe male	No genital tassel Black body Broad red dorsal and caudal fin margin	Genital tassel	Silvery body Pale head mask from eyes to snout (white, blue, green to silver in colour) (Figure 3.3)	Black body Southern males may have a yellow dorsal fin margin (white in other males)	Very black body White dorsal and caudal fin margin. (Figure 3.2)
Ripe female				No stripes Coppery body Slightly dark on head or totally dark when ripe	Vertical bars distinct Darker head than *O. karongae* Black stripe between eyes
Anal spines	4	3			
Jaws	Short			Short and thick	Long and large
Teeth			Clear front row followed by mixed array	4/5/6 up to 15 rows Overlapping	3 to 5 rows Clear cut
Snout shape	Quite pointed	Rounded			
Body shape	Deep flat body Arched head		Wide middle Fat and rounded	Flat squat body Shorter operculum than *O. lidole*	Tapering, emaciated appearance Very large operculum

The number of samples and tissue types collected for allozyme analysis was limited by the amount that could be held in one liquid nitrogen dry shipper. Tissue samples were obtained as soon as possible to avoid tissue deterioration. A small piece of muscle tissue was cut from the body wall, and a sample of liver tissue taken and immediately frozen in liquid nitrogen. A fin clipping was taken from the caudal fin and preserved in 20% DMSO salt-saturated solution for DNA analysis (Chapter 4). Morphological measurements, assessments of sex and maturity were also made (Chapter 5).

3.2.1.1 Sample sites of wild fish

Fish were purchased from local fishermen at villages on the western shore of the SE arm of Lake Malawi (Macawa), and on the western shore of Lake Malombe (Chimwala) (Table 3.2 and Figure 3.5). Fish were collected by the local fishermen using gill nets in the shallow waters close inshore. *O. squamipinnis* was largely collected from Lake Malombe, where it matures earlier in the season than in Lake Malawi. *O. shiranus* was found only in catches from Lake Malombe, although samples (fin tissue only) were also taken from a garden pond at Chirombo Bay (property of Mr and Mrs Ndunes). Samples of *O. karongae* were also collected from the garden pond. These fish were regarded as representative of the wild population since the pond had only recently been stocked and there was no reason to suspect that the fish were hybridizing (primarily based on morphological characteristics and later confirmed with molecular genetic analysis). Other fish were collected from relatively deeper waters by offshore trawling; these were purchased from the

Table 3.2 Location and size of *Oreochromis* fish samples collected from the wild and farm ponds. Number of fin tissue samples collected for RAPD analysis (Chapter 4) and frozen tissue (muscle and liver) for allozyme analysis.

Sample site	Locality		Fin *	Frozen *
Wild sites		Species		
Lake Malombe	Chimwala (I)	*O. squamipinnis*	15 (20)	20
		O. shiranus	3 (9)	9
		Unknown	3	3
	Chimwala (II)	*O. shiranus*	10 (12)	11(12)
Lake Malawi	South of Boadzulu Island	*O. squamipinnis*	0 (3)	3
		O. lidole	0 (10)	10
		O. karongae	8 (25)	14 (17)
	Macawa	*O. karongae*	1 (7)	7
	Maldeco fisheries	*O. lidole*	4 (8)	8
		Unknown	4 (8)	7
Chirombo Bay	Garden pond	*O. shiranus*	7 (8)	-
		O. karongae	12	-
Farm sites		Abbreviation		
Domasi	*O. karongae* pond	DMOK	15	9 (10)
	O. shiranus pond	DMSH	15	10
Dwangwa	Sewage tank pond	DWSE	27 (30)	30
	Storage tank pond	DWST	18 (50)	47 (50)
Mzuzu	*O. shiranus* holding pond	MZSH	15	-
	O. karongae holding pond	MZKH	10	10
	O. karongae breeding pond	MZKB	18	-

* Figure in parenthesis indicates number of samples originally collected, if different from the number analysed successfully.

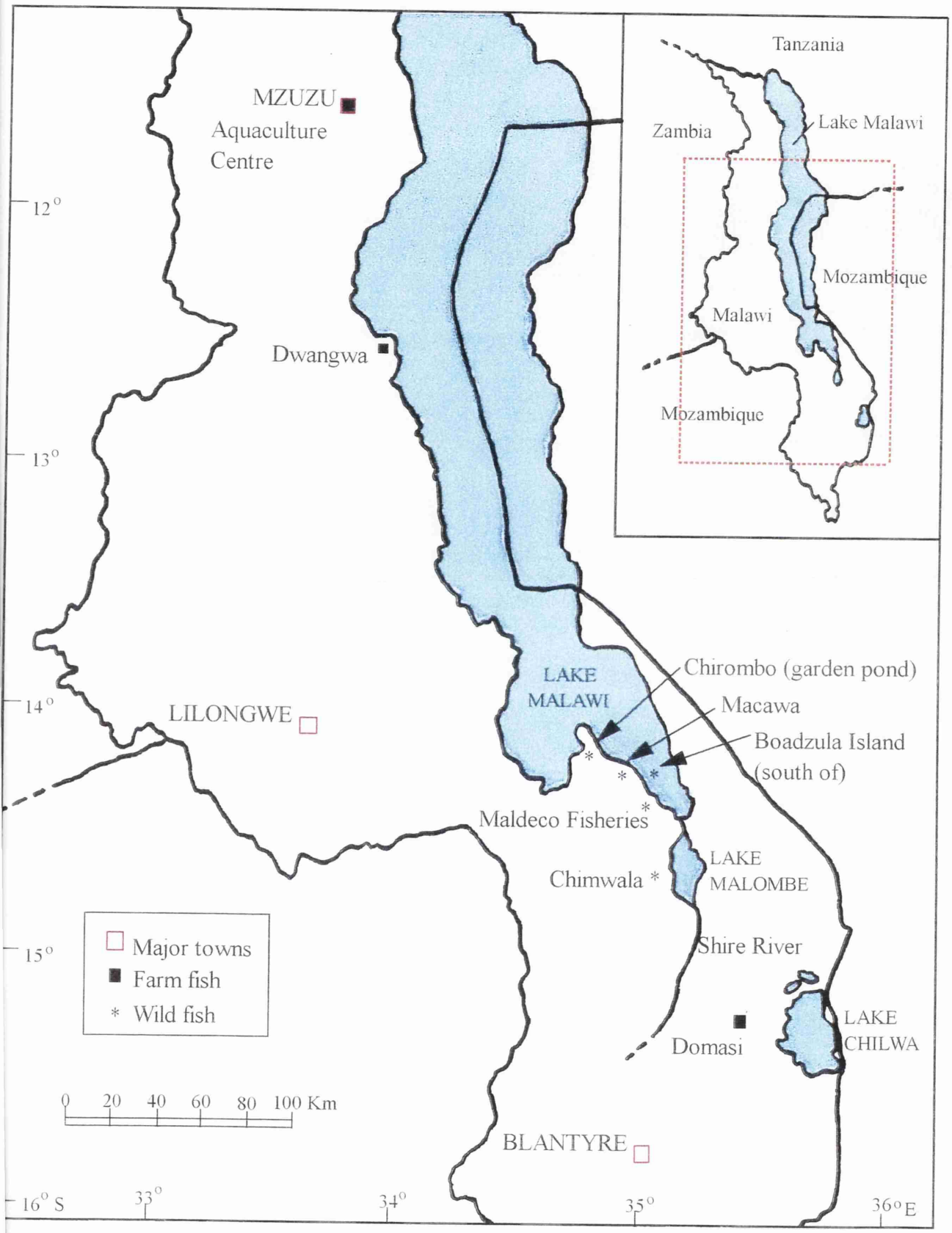


Figure 3.5 Sampling sites of farm and wild fish in Malawi. Insert shows countries bordering Malawi, and the section enlarged in the main figure.

commercial fisheries at Maldeco and collected on the Monkey Bay research vessel (the 'Ndunduma'). Trawling on the Ndunduma was carried out south of Boadzulu Island (Figure 3.5), in two catches. The first trawl, containing fish identified as *O. lidole*, *O. karongae* and a few as *O. squamipinnis*, was for 30 minutes across smooth substrate at a maximum depth of 34 m. The second trawl, where only *O. karongae* was identified, was at a shallower maximum depth of 10 m.

3.2.1.2 Sample sites of farm fish

Samples of fish were collected from three farms in Malawi:

1) *Southern Regions Aquaculture Centre, Domasi.* An experimental fish farm set up by ICLARM and the Malawi Fisheries Department, with input from the University of Malawi (Chancellor College, Zomba). Tissue samples were taken from fish in a pond thought to be stocked with *O. shiranus* (DMSH) and a pond thought to be stocked with *O. karongae* (DMOK).

2) *Dwangwa Sugar Corporation Ltd.* A commercial sugar cane plantation that started to stock tilapia after they were unintentionally pumped in from the lake with irrigation water. Samples of fish were taken from two ponds; a culture (sewage) pond (DWSE) stocked with unidentified fish and fertilized with human sewage, and a water storage pond (DWST) that was intentionally stocked with chambo taken from the sewage pond.

3) *Central and Northern Regions Aquaculture Centre, Mzuzu.* An experimental aquaculture facility set up by the EC / Stirling University Project (finished 1995), that supplied tilapia to small-scale farmers in the northern region of Malawi. Tissue samples for allozyme and RAPD analysis were taken from an *O. karongae* holding pond (MZKH). Samples for RAPD analysis only, were taken from an *O. shiranus* holding pond (MZSH) and an *O. karongae* breeding pond (MZKB).

3.2.2 Observations on pond conditions

To assess the effect of culture conditions on the incidence of hybridization, several observations were made during sampling, including pond size and turbidity (using a secchi disc), the species and sex ratios, and the stocking density and stocking history of the pond concerned (when available). Information on stocking density and pond size may provide some indication of the size of nesting area available. The size of fish and the occurrence of actively mouthbrooding females were also noted to ascertain how vigorously the fish were breeding. Details on the incidence of flooding and the proximity of a river or stream to the farm ponds were noted to assess the possible avenues for escape of farm fish and hybrids (if found).

3.2.3 Laboratory Procedures

Standard starch gel electrophoresis (Harris & Hopkinson 1976; Hillis & Moritz 1990; Ferguson 1985) was employed to screen for documented (Sodsuk *et al.* 1995) and other possible diagnostic loci of *O. shiranus* and chambo. After some initial screening, all samples were analysed at 12 enzymes, representing 13 loci (Table II.1, Appendix II).

Small pieces of muscle or liver tissue were removed by a scalpel from the frozen samples and homogenised manually with a glass rod, with 25 μl of 0.1 M Tris HCL (pH 7.5) in 0.5 cm perspex wells. The crude homogenate was absorbed directly into 3 x 6 mm pieces of Whatman No.1 filter paper, and blotted to remove excess liquid. In order to monitor the progress of the run, two inserts stained with ferritin were placed at either end of the origin. Gels were run at 4°C in a refrigerator, for 4 to 5 hours. After electrophoresis, gels were sliced horizontally into three 2 mm slices. The cut surface was stained and incubated in the dark at 37°C until bands appeared - usually within one hour. Esterase however, stained almost immediately at room temperature in the light, and bands were only visible over a U.V. light source. Stain recipes (Table II.3, Appendix II) were modified from Aebersold *et al.* (1987), Ferguson (1985), and Murphy *et al.* (1990). Enzyme, locus and allele nomenclature follow those of Shaklee *et al.* (1990). Samples were run next to each other to clarify allele identities, as assigned by Sodsuk *et al.* (1995), by using *Oreochromis mossambicus* alleles as standards.

3.2.4 Data Analyses

The BIOSYS-1 (Swofford & Selander 1981) computer package was used to estimate allele frequencies and measures of genetic diversity for the pure species and farm pond populations sampled. Hybrids were identified in the farm pond populations using species diagnostic loci. The POPGENE (Yeh & Boyle 1996) computer package was also used to estimate a measure of gene diversity, based on Shannon's (1949) Information index. Detection of genotypic linkage disequilibrium, deviations from Hardy-Weinberg equilibrium and genetic differentiation between populations (testing if allelic composition is independent of population assignment) were tested using Fishers' exact test, using the GENEPOP (Raymond & Rousset 1995) computer package. Genetic differentiation between sub-samples of the wild species was tested for using the same procedure. The sequential Bonferroni test (Rice 1988) was applied to tables of multiple statistical tests to correct for the occurrence of results significant by chance. Species differences were also measured by calculating F_{ST} (Weir & Cockerham 1984), and its significance tested by a modified

Fishers' exact test (GENEPOP, Raymond & Rousset 1995). The probability of the observed distribution of genotypes (based on diagnostic loci only) at all farm populations occurring by chance alone, was estimated by Monte Carlo simulations (10,000 replicates) of all pairwise combinations of farm ponds (REAP, McElroy *et al.* 1991).

Because sample sizes were relatively small, and from populations that were of varying sizes and founded with a different number of individuals (i.e. wild and farm), three methods were used to estimate distances between populations. Distances were used to construct dendrograms according to the nearest neighbour-joining method (Saitou & Nei 1987), and compared for any differences in the relative positions of populations. The PHYLIP (Felsenstein 1993) computer package was used to estimate genetic distances between samples, according to Reynolds, Weir and Cockerham's (1983) genetic distance and Cavalli-Sforza's chord measure (Cavalli-Sforza & Edwards 1967). Unlike Nei's genetic identities, the two genetic distances used do not assume that population sizes have remained equal and constant in all populations, and is thus appropriate to evaluate genetic differentiation of the farm populations. Allele frequencies were bootstrapped over loci 1000 times and a consensus dendrogram constructed to estimate the confidence to be placed on the nodes of the dendrogram to ascertain the robustness of the data. The NTSYS (Rohlf 1992) computer package was used to compute dissimilarity coefficients between samples from the gene frequency data, according to the average Manhattan distance. The average Manhattan distance coefficient is commonly used as a measure of dissimilarity in numerical taxonomy and for population classification, it has no genetic assumptions and may therefore be more appropriate than the previous two measures in inferring relationships between the wild and farm pond populations.

3.3 RESULTS

3.3.1 Pond Observations

Details given by staff on the stocking history of ponds at each farm were generally very 'sketchy', apart from the Aquaculture centre at Mzuzu where the information provided was relatively detailed. All farms appeared to be ineffective in controlling promiscuous breeding because the fish sampled from individual ponds varied greatly in size. The ponds sampled from the experimental farms (Domasi and Mzuzu) were supposedly stocked with either *O. karongae* or *O. shiranus,* presumably to conduct experiments on their potential growth rates under different regimes. There were no indications that any method apart from using morphological characters, was used to identify fish while stocking. No efforts were made to identify fish when the DWSE

pond at Dwangwa was originally stocked, although *O. karongae*-type fish (based on morphology) were taken from the DWSE to stock the DWST pond (A. Brooks *pers comm.*). Details on observations made at each farm are shown in Table 3.3. Putative hybrids were suspected mainly on the basis of individuals found which had the colouration and body shape of *O. shiranus* but only

Table 3.3 Details of pond size, Secchi disc turbidity reading, stocking history and general observations at fish farm ponds sampled in Malawi. For locations of pond sites see Figure 3.5, for photographs of ponds see Figure 3.6-3.11.

Pond✡	Size	Turbidity	Spp. originally stocked & source	Other observations
DMOK	900 m^2 1m deep	21.5 cm	*O. karongae:* Lake Chilwa and Lake Malawi	Putative hybrids* Identification uncertain, but indications of all chambo species present, also *Tilapia rendalli* and other *Oreochromis* species present (possibly *O. mossambicus*)
DMSH	500 m^2 1m deep	22.5 cm	*O. shiranus shiranus*: Lake Malawi and Upper Shiré river. *O. shiranus chilwa*: Lake Chilwa	Putative hybrids* Fish appeared to resemble *O. shiranus*
DWSE	9000 m^2 1m deep	**	Restocked annually with approximately 5 tonnes fish, mainly *O. shiranus* but identification largely unknown: Irrigation water from Lake Malawi	Many putative hybrids* Mainly *O. shiranus,* but also 'chambo-type' fish present. Many mouthbrooding females. Unusual body shapes, stunted appearance and jaw deformities.
DWST	200 m^2	60.5 cm	Stocked in 1994 with chambo species, tried to stock only *O. karongae:* Irrigation water and DWSE pond	Putative hybrids* Mainly 'chambo-type' fish. Unusual body shapes, stunted appearance and jaw deformities.
MZKH	150 m^2	21.5 cm	1991-1992 *O. karongae*: Nkata Bay, Karongae Bay and Monkey Bay (Lake Malawi)	Putative hybrids* Mainly identified as *O. karongae.*
MZKB	300 m^2	21.0 cm	Species & source as for MZKH 1 fish per m^2 # sex ratio 1 : 2 †	No indications of hybrids* Mainly identified as *O. karongae.*
MZSH	300 m^2	21.5 cm		No indications of hybrids* Mainly *O. shiranus*, but also *O. karongae* present

✡ DMOK, *O. karongae* pond Domasi (Fig. 3.6); DMSH, *O. shiranus* pond, Domasi (Fig. 3.7); DWSE, Sewage pond Dwangwa (Fig. 3.8); DWST, Storage tank pond Dwangwa (Fig.3. 9); MZKH, *O. karongae* holding pond Mzuzu; MZSH, *O. shiranus* pond Mzuzu; MZKB, *O. karongae* breeding pond Mzuzu (Fig. 3.11).

* Putative hybrids suspected if individualswere found with the colouration and body shape of*O. shiranus* but with only three anal fin spines (indicative of chambo species), or some other characters of both species (see Table 3.1).

** Turbidity reading not taken, but water clarity was extremely poor.

Actual density of fish probably lower due to predation.† Sex ratio - male : female.

three anal fin spines (indicative of chambo species) (discussed further in Chapter 5). Additional information and characteristics of the farm ponds studied are presented below:

1) *Southern Regions Aquaculture Centre, Domasi* (Figures 3.6 and 3.7). In both ponds the fish were densely populated, water clarity was poor and putative hybrids were identified. Both *O. shiranus* and chambo fish were found in the two ponds sampled, although each pond was dominated by the species that had intentionally been stocked (*O. shiranus* in DMSH, and *O. karongae* in DMOK). The presence of non-endemic species of tilapia was also suspected in the DMOK pond (Table 3.3). Fish from the centre have been used to restock Lake Chilwa when it has dried out. *O. shiranus chilwa* have also been used to stock dams (1950s - 1970s) on Lilongwe river (Kamuzu dam, Masula dam, Mbabzi estate), and chambo have been distributed to farms around the Zomba plateau. A small stream was found to run near the farm, which may act as an avenue for escape during flooding.

2) *Dwangwa Sugar Corporation Ltd.* (Figure 3.8). The stocking of tilapia at the plantation was originally unintentional, and therefore no efforts were made to separate the species or to stock a particular one. Fish resembling both species were found in the sewage (DWSE) pond, although *O. shiranus* looking fish were dominant. Attempts were made in 1994 to stock only *O. karongae* in the storage tank (DWST) pond (Figure 3.9), and the fish sampled mainly resembled chambo. Putative hybrids were found in both ponds. The stocking density at all ponds is unknown, although during sampling it became obvious that the ponds were densely populated, more so than the ponds sampled at the other farms, and the fish were breeding prodigiously. The size of fish varied greatly (most less than 10 cm long) and many females were found to be carrying mouthbroods. The sewage pond was richly fertilized, water clarity was poor and many fish were full of food (noticed when sexing). Many fish had deformed jaws and body shapes unlike those of chambo or *O. shiranus,* with large eyes, an unusual head shape and stunted appearance. The fish from the sewage pond were originally used for human consumption (from 1978) but from 1985 they have been used as feed at a crocodile farm on the plantation. Fish stocks are supplemented by annual restocking (Table 3.3). Since the founder fish originally entered via the irrigation water it is highly likely that there are avenues for escape back to Lake Malawi, within close proximity to the plantation.

3) *Central and Northern Regions Aquaculture Centre, Mzuzu* (Figure 3.10). The centre practices polyculture of the catfish *Clarias gariepinus* with *O. shiranus* or *O. karongae,* and presently concentrates on farming *O. shiranus* because it is easier to breed. Water quality was poor in all the ponds sampled. Putative hybrids were found in the *O. karongae* holding pond only (MZKH;

Figure 3.6 *O. karongae* pond, Domasi (DMOK) - only a narrow path separates the ponds.

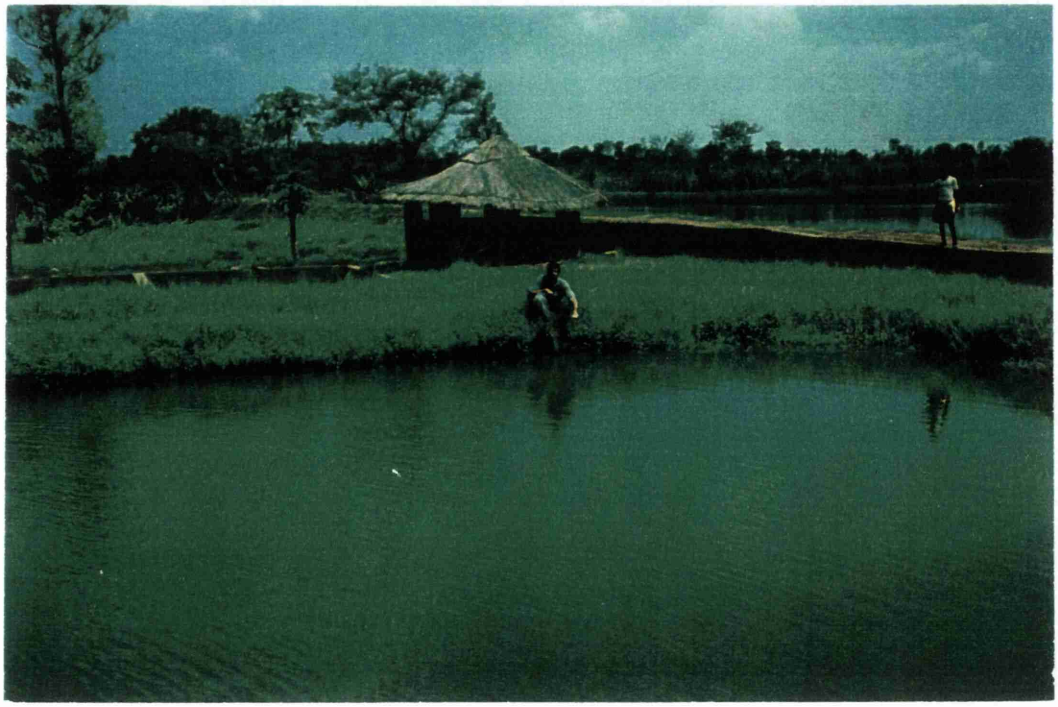


Figure 3.7 *O. shiranus* pond, Domasi (DMSH) (note that the picture is greener than in reality).

Figure 3.8 Sampling at the Sewage pond, Dwangwa sugar cane plantation (DWSE).

Figure 3.9 Storage tank pond, Dwangwa sugar cane plantation (DWST).

Figure 3.10 *O. karongae* breeding pond, Mzuzu (MZKB).

Figure 3.11 Closely arranged fish ponds at Mzuzu.

Figure 3.11), although more than one species was also found in the *O. shiranus* pond (MZSH). The farm suffered from severe flooding in April 1991, after which the fish were separated again (using morphological characteristics to identify species). A small stream was found to run near the farm, which may act as an avenue for escape during flooding. Severe otter predation has decreased numbers of fish (of all species) and there is also a problem due to proliferations of the *Xenopus* tadpole. Apart from the adults eating tilapia fry, the tadpoles also compete with the fish for space and nutritional resource, exemplifying the problems associated with overstocking.

3.3.2 Allozyme polymorphism

Initial screening of 15 enzymes with three buffer systems (Table II.1, Appendix II) revealed 12 enzymes with sufficient activity and resolution to be used for further analyses (Table 3.4). Of the 13 loci analysed, 11 were polymorphic in one or more of the four species and five pond populations tested (Table II.4, Appendix II). The alleles detected at all loci are generally the same as those published for chambo and *O. shiranus* (Sodsuk *et al.* 1995), although a larger number of alleles were detected in the present study, particularly for chambo species at the loci *ADA**, *IDDH**, *PGM** and *EST** (Table II.4, Appendix II).

Table 3.4 Enzymes scored, with their common abbreviation, EC number, number of loci, number of alleles scored at polymorphic loci (P 0.95) (/ monomorphic). (Examples of gels: Figures II.1-II.4, Appendix II).

Enzyme	Abbreviation	Enzyme no.	no. of loci	no. of alleles
Adenosine deaminase	ADA	2.6.1.1	1	8
Alanine aminotransferase	ALAT	2.6.1.2	1	3
Creatine kinase	CK	2.7.3.2	1	3
Esterase	EST	3.1.1.-	1	4
Glucose-6-phoshate isomerase	PGI	5.3.1.9	1	3
L-Iditol dehydrogenase	IDDH	1.1.1.14	1	6
Isocitrate dehydrogenase	IDH	1.1.1.42	1	/
L-Lactate dehydrogenase	LDH	1.1.1.27	2	2,2
Malate dehydrogenase	MDH	1.1.1.37	1	/
Phosphogluconate dehydrogenase	PGDH	1.1.1.44	1	2
Phosphoglucomutase	PGM	5.4.2.2	1	5
Superoxide dismutase	SOD	1.15.1.1	1	2
Total			13	40

Among the chambo species, seven loci were polymorphic at the 95% level. The three chambo species shared three of these loci (*ADA**, *LDH-1** and *SOD**). *O. squamipinnis* alone was polymorphic at *EST**, and like *O. karongae* was polymorphic at *PGM**, for a total of five polymorphic loci. *O. lidole* like *O. karongae* was polymorphic at *IDDH**, and was also variable at *PGDH**, making both species polymorphic at a total of five loci. The 'unknown' fish could only be identified as chambo, and were not used further in this study. A number of species-specific alleles, at low frequencies, were observed in *O. squamipinnis* (*ADA*104, EST*93, EST*77, PGM*130* and *PGM*45*) . *O. karongae* and *O. lidole* shared a number of alleles not found in *O. squamipinnis* (*ADA*80, IDDH*120* and *IDDH*35*).

O. shiranus was polymorphic at five different loci (*ADA*, IDDH*, PGDH*, PGM** and *SOD**). Four of the loci analysed were species diagnostic, and could therefore be used to distinguish between *O. shiranus* and all of the chambo species (*ADA*, ALAT*, CK** and *IDDH**). Two of these were monomorphic (*ALAT*100*, and *CK*115*) and the other two were polymorphic (*ADA*70*, *ADA*60* and *IDDH*100*, *IDDH*20*).

The farm pond populations shared three polymorphic loci (*ADA*, PGDH** and *SOD**). All alleles found in *O. shiranus* and/or the chambo species, except *ADA*104*, were present in one or more of the farm ponds sampled. In four of the farm ponds a number of alleles were found that had not been detected in either the chambo species or *O. shiranus*. Of these alleles, Sodsuk *et al.* (1995) observed *LDH*180* in *O. shiranus*, and the *PGI** alleles only in other *Oreochromis* species (*PGI*115* in *O. mossambicus*, *O. mortimeri* and *O. andersonii*; *PGI*89* in *O. placidus*). The occurrence of these generally rare 'unique' alleles varied, although higher frequencies were detected in the DWST pond, where the greatest number of alleles were found which had not been detected in either chambo species or *O. shiranus* (Table 3.5).

Table 3.5 Alleles unique to farm pond populations (i.e. not detected in either the chambo species or *O. shiranus*) and their respective frequencies.

Allele	Farm pond†			
	DMSH	DMOK	DWSE	DWST
*ALAT*60*				0.213
*CK*67*		0.056		
*EST*108*	0.150	0.056		0.074
*IDDH*150*			0.083	0.011
*LDH*180*	0.150		0.033	0.734
*PGI*115*	0.150			0.691
*PGI*89*				0.022

† DMSH, *O. shiranus* pond Domasi; DMOK *O. karongæ* pond Domasi; DWSE Sewage pond Dwangwa; DWST Storage tank pond Dwangwa.

3.3.3 Incidence of hybridization

Assigning whether an individual was or was not a hybrid was based on the four diagnostic loci (*ADA**, *ALAT**, *CK** and *IDDH**). Since it was not possibly to separate *O. squamipinnis*, *O. karongae* and *O. lidole*, individuals possessing alleles found only in these species will be referred to as chambo. Hybrids between chambo and *O. shiranus* were found in all farm ponds (Table 3.6). The highest frequency of hybrids was found in samples from the *O. shiranus* pond at Domasi and the two ponds at Dwangwa. No ponds contained more than one type of 'pure' species (i.e. *O. shiranus* or chambo).

Table 3.6 Frequency of chambo, *O. shiranus* and hybrid fish in farm ponds, based on four diagnostic loci.

Farm pond☼	no.	Chambo (%)	*O. shiranus* (%)	hybrids (%)
DMSH	10	0.0	30.0	70.0
DMOK	9	77.8	0.0	22.2
DWSE	30	0.0	26.7	73.3
DWST	47	8.5	0.0	91.5
MZKH	10	70.0	0.0	30.0

☼ DMSH, *O. shiranus* pond Domasi; DMOK *O. karongae* pond Domasi; DWSE Sewage pond Dwangwa; DWST Storage tank pond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu.

The frequencies presented in Table 3.6 are the minimum frequency of hybrids, since individuals with genotypes of pure chambo or *O. shiranus*, may in fact be later generation hybrids. This is highly likely in the DMSH, DWSE and DWST ponds where hybridization is extensive (70% or more of the individuals sampled). In all of the farm ponds sampled, no F_1 hybrids (i.e. individuals heterozygous at all diagnostic loci) were found. Hybrids were therefore progeny from a backcross to *O. shiranus*, backcross (BC) to chambo, or a later generation hybrid (F_1 hybrid x F_1 hybrid, BC hybrid progeny x F_1 hybrid or BC x BC).

Allele frequencies for samples from both ponds at Domasi reflect the species (*O. shiranus* and chambo) that were chosen for stocking (Table 3.7). The *O. shiranus* pond sample (DMSH) was dominated by *O. shiranus* alleles; all hybrids were homozygous for *O. shiranus* alleles at two or more of the four diagnostic loci (Table II.5, Appendix II). The two hybrids found in the *O. karongae* pond sample (DMOK) were later generation hybrids, both homozygous for chambo alleles at two or more diagnostic loci.

The sample from the sewage pond at Dwangwa (DWSE) was dominated by *O. shiranus* alleles. All hybrids were homozygous for *O. shiranus* alleles at two or more loci, and only two individuals were homozygous for chambo alleles at two loci. The storage pond sample (DWST), where *O. karongae* fish had been stocked (taken from the sewage pond), was largely dominated by chambo alleles. Over 76% of hybrids were homozygous for chambo alleles at two or more

Table 3.7 Cumulative frequencies of alleles at *O. shiranus* (SH) and chambo (CH) diagnostic loci, at each farm pond tested, illustrating the relationship between the species chosen for stocking and the dominance of alleles for that particular species (SH or CH).

Locus	Species	Diagnostic allele(s)	DMSH	DMOK	DWSE	DWST	MZKH
*ADA**	SH	*60, 70*	0.600	0.000	0.850	0.128	0.000
	CH	*80, 93, 100, 104, 108, 118*	0.400	1.000	0.150	0.872	1.000
*ALAT**	SH	*100*	1.000	0.222	1.000	0.213	0.250
	CH	*87*	0.000	0.778	0.000	0.574	0.750
*CK**	SH	*115*	1.000	0.056	1.000	0.649	0.000
	CH	*100*	0.000	0.888	0.000	0.351	1.000
*IDDH**	SH	*20, 100*	0.900	0.000	0.433	0.405	0.000
	CH	*35, 70, 120*	0.100	1.000	0.484	0.584	1.000

diagnostic loci (Table II.5, Appendix II). As well as later generation hybrids (already discussed), the number of hybrids found in the DWSE and DWST pond samples increases (to 86.7% and 93.6%, respectively) if non-diagnostic loci are also employed. For example, five individuals that are homozygous for all *O. shiranus* diagnostic alleles, also have the allele *EST*93* or *EST*77*, found otherwise only in *O. squamipinnis*, and could therefore represent later generation *O. shiranus* backcross hybrids.

The sample from the *O. karongae* holding pond at Mzuzu (MZKH), contained largely 'pure' chambo, and all three hybrids were homozygous for chambo alleles at three of the four diagnostic loci (Table II.5, Appendix II). This pond was the only one that did not contain alleles unique to *O. squamipinnis*. Since *O. karongae* and *O. lidole* could not be distinguished, it is not possible to say whether both or one of the two species was present based on allozyme data alone, although morphological data suggests that *O. karongae* was the only chambo species present in the MZKH pond (see Chapter 5).

3.3.4 Deviations from Hardy-Weinberg expectations

The four species sampled from the wild deviated from Hardy-Weinberg equilibrium (HWE) at seven loci, out of the 20 possible tests. All F_{is} values were positive, indicating that the deviations from HWE were due to a deficiency of heterozygotes (see Table 3.10). In combinations over all loci, three of the four species showed significant deviations from HWE; *O. karongae*, *O. lidole* and *O. shiranus* (Table 3.8). All wild species were sampled from more than one site; *O. shiranus* and *O. squamipinnis* from different lakes. For *O. shiranus* and *O. lidole,* the allele *PGDH*116*

(homozygous condition) was found in only one of the two sites where fish were sampled from; Chimwala II and Maldeco, respectively. Two alleles of the *ADA** locus (*ADA*100* and *ADA*118*) were found in only the Maldeco sample of *O. lidole*. *ADA*118* was restricted to one of the sample sites of *O. karongae* (Boadzulu Island). The *PGM** locus was not found to deviate significantly from HWE, although *PGM*160* was found in only the *O. shiranus* sample from Chimwala I.

Deviations from Hardy-Weinberg equilibrium at individual loci, were found in all of the farm pond samples (Table 3.8). Significant deviations were found in combinations over all loci for the Dwangwa (DWSE and DWST) and Mzuzu (MZKH) samples. Deviations at individual loci for the samples from Domasi (DMSH and DMOK) were no longer significant after the sequential Bonferroni test (5% significance level) was applied. All F_{IS} values were positive, indicating that the deviations from HWE were due to a deficiency of heterozygotes. Deficiencies of heterozygous genotypes were found at all diagnostic loci except CK*, and all farm pond samples had at least one diagnostic loci deviating from HWE.

Table 3.8 Significant deviations from Hardy-Weinberg equilibrium (Fishers method) in combinations over all loci and for individual loci, in each species and farm population sampled.

Sample	Over all loci			At individual loci		
	chi^2	*df*	*P*	loci †	*P*	F_{IS}
OSHI	21.5	10	*	*PGDH**	***	1.00
OSQU	16.0	10	n.s	*LDH-1**	**	0.78
OKAR	37.4	10	***	*LDH-1**	***	0.89
				*ADA**	***	0.56
OLID	49.7	10	***	*LDH-1**	***	1.00
				*ADA**	***	0.53
				*PGDH**	***	1.00
DMSH	20.0	14	n.s	(*ADA**	*	0.26)
				(*PGDH**	**	1.00)
DMOK	12.4	14	n.s	(*ALAT**	*	1.00)
DWSE	Infinity	14	****	(*ADA**	*	0.11)
				*PGDH**	***	0.89
				*IDDH**	***	0.27
DWST	Infinity	22	****	*ALAT**	***	0.71
				*SOD**	***	0.74
				*PGI**	***	0.38
				*LDH-1**	*	1.00
				*LDH-2**	***	0.52
				*PGM**	***	0.59
				*ADA**	***	0.57
				*PGDH**	***	1.00
				*IDDH**	***	0.46
MZKH	25.6	12	*	(*ALAT**	*	0.76)
				*PGDH**	***	1.00

OSHI, *O. shiranus*; OSQU, *O. squamipinnis*; OKAR, *O. karongae*; OLID, *O. lidole*; DMSH, *O. shiranus* pond Domasi; DMOK *O. karongae* pond Domasi; DWSE Sewage pond; DWST Storage tank pond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu.
chi^2, chi-squared value; *df*, degrees of freedom; *P*, significance where: n.s, not significant; *$P<0.05$; **$P<0.01$; ***$P<0.005$; ****$P<0.001$; † Results in parenthesis are no longer significant after the sequential Bonferroni test is applied (5% level).

3.3.5 Linkage disequilibria

Of the 173 tests carried out, significant linkage disequilibria was observed only in samples from the DWSE pond (3 out of 21 tests) and DWST pond (8 out of 55 tests) at Dwangwa (Table 3.9). If the sequential Bonferroni test is applied (5% level) all tests for the DWSE pond are non-significant, and only four of the eight tests from the DWST pond sample remain significant. However, these remaining significant results indicate that alleles at a number of loci in the DWST pond population are not in linkage equilibrium.

Table 3.9 Loci at which significant linkage disequilibria was observed, in samples from the DWSE and DWST ponds at Dwangwa.

Sample	locus 1	locus 2	*P* †
DWSE	*PGDH**	*SOD**	(0.0203)
	*PGDH**	*ADA**	(0.0108)
	*IDDH**	*SOD**	(0.0055)
DWST	*LDH-2**	*PGI**	<0.0001
	*CK**	*PGI**	0.0005
	*CK**	*LDH-2**	0.0007
	*PGM**	*PGI**	0.0007
	*PGM**	*CK**	(0.0353)
	*ADA**	*PGI**	(0.0132)
	*ADA**	*LDH**	(0.0087)
	*ADA**	*EST**	(0.0250)

† probability of significant result: results in parentheses are no longer significant after the sequential Bonferroni test is applied (5% significance level).

3.3.6 Genetic diversity within wild and farm pond samples

It was not possible to identify which species of chambo were present in farm pond samples, and the fish identified as 'pure' chambo or *O. shiranus* may actually be later generation hybrids (see section 3.3.3, and section 4.3.5 in Chapter 4). Furthermore, sample sizes of 'pure' fish from farm pond populations were small (eight or less, see Table 3.6). Therefore, measures of genetic diversity were estimated for the farm pond populations as a whole, rather than for each species and hybrid population individually.

A deficit of heterozygotes were found in all wild and farm pond samples (Table 3.10), reflecting the results of deviations from Hardy-Weinberg equilibrium (Table 3.8). The mean number of alleles per locus and the expected levels of heterozygosity were similar for the three chambo species (standard errors were high in some cases). The mean number of alleles per locus was lower for *O. shiranus* than for chambo, although the level of heterozygosity was higher in this species. The percentages of polymorphic loci were the same for all wild species.

As expected, due to the presence of more than one species and the 'unique' alleles found, all measures of genetic diversity were higher in each farm pond (except MZKH), than in the wild species. The highest values were for DWST, where the greatest number of alleles (not present in

the wild populations) and hybrids were found. No alternative alleles were found in the MZKH pond and there was a relatively low number of hybrids. Mean measures of gene diversity, based on Shannon's (1949) Information Index, compare well with the other measures, although the diversity for *O. shiranus* is very similar to the chambo species for this measure of genetic diversity.

Table 3.10 Genetic diversity observed in wild species and farm pond samples; mean number of alleles per locus, observed and expected heterozygosities, percentage of polymorphic loci and a mean measure of genetic diversity loci based on Shannon's (1949) Information Index (standard error in parenthesis).

Sample †	no.	Mean no. of alleles	Heterozygosity		% Polymorphic loci	Shannon Index (mean)
			Direct count	H-W expected		
OSHI	20	1.38 (0.14)	0.096 (0.042)	0.137 (0.055)	38.46	0.205 (0.287)
OSQU	23	1.85 (0.37)	0.087 (0.040)	0.112 (0.050)	38.46	0.212 (0.340)
OKAR	21	1.69 (0.33)	0.051 (0.022)	0.107 (0.049)	38.46	0.194 (0.319)
OLID	18	1.69 (0.03)	0.060 (0.036)	0.122 (0.050)	38.46	0.208 (0.295)
DMSH	10	1.92 (0.35)	0.162 (0.056)	0.192 (0.064)	53.85	0.345 (0.444)
DMOK	9	1.85 (0.27)	0.137 (0.049)	0.182 (0.057)	53.85	0.309 (0.346)
DWSE	30	2.15 (0.45)	0.151 (0.061)	0.194 (0.075)	53.85	0.364 (0.530)
DWST	47	3.00 (0.31)	0.180 (0.049)	0.332 (0.076)	84.62	0.605 (0.537)
MZKH	10	1.62 (0.24)	0.085 (0.042)	0.154 (0.054)	46.15	0.254 (0.326)

† OSHI, *O. shiranus*; OSQU, *O. squamipinnis*; OKAR, *O. karongae*; OLID, *O. lidole*; DMSH, *O. shiranus* pond Domasi; DMOK *O. karongae* pond Domasi; DWSE Sewage pond Dwangwa; DWST Storage tank pond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu.

3.3.7 Genetic differentiation between wild sample sites

Over all loci, no significant genetic differentiation was observed between separate sample sites for each of the wild species sampled (see Table 3.2 for sites). At individual loci, only one significant result was found between the *O. shiranus* samples (Chimwala I and Chimwala II) for the locus *PGM** (P=0.013). However, this result was not significant under the Bonferroni test. No significant F_{ST} values (ranging from −0.077 to 0.021) were observed between separate sample sites for each of the wild species sampled.

3.3.8 Genetic differentiation between all populations sampled

The allelic composition of each farm pond sample as a whole, rather than that of the individual species or hybrid sub-populations within each sample, were used in the analysis of genetic differentiation between populations (reasoning as in section 3.3.6). Allelic composition at all polymorphic loci was significantly different across all nine (wild and farm) populations

(Fisher's method, test combination: chi^2 = Infinity, df = 22, P<0.001). All pairwise comparisons of populations (wild species and farm ponds) were significant over all loci, except between samples from the Domasi *O. karongae* pond (DMOK) and Mzuzu *O. karongae* holding pond (MZKH) (Table 3.11 and Table II.6, Appendix II).

Table 3.11 Significance of genetic differentiation, over all loci, between all pairs of populations sampled (See Table II.6 Appendix II, for details of individual loci showing significant differentiation in allelic composition in each pairwise comparison).

	OSHI	OSQU	OKAR	OLID	DMSH	DMOK	DWSE	DWST
OSQU	****							
OKAR	****	****						
OLID	****	***	*					
DMSH	***	****	****	****				
DMOK	****	**	*	*	****			
DWSE	****	****	****	****	****	****		
DWST	****	****	****	****	****	****	****	
MZKH	****	***	**	***	****	n.s	****	****

OSHI, *O. shiranus*; OSQU, *O. squamipinnis*; OKAR, *O. karongae*; OLID, *O. lidole*; DMSH, *O. shiranus* pond Domasi; DMOK *O. karongae* pond Domasi; DWSE Sewage pond Dwangwa; DWST Storage tank pondDwangwa; MZKH, *O. karongae* holding pond Mzuzu; * P<0.05; ** P<0.01; *** P<0.005; **** P<0.001; n.s, Not significant.

Across all loci, all chambo species differed highly significantly from *O. shiranus* in allelic composition (Table 3.11), and a relatively high F_{ST} value of 0.527 (P<0.001) was observed. In comparison, a very low level of genetic differentiation was observed between the three chambo species alone: F_{ST} = 0.067 (P<0.005). Nevertheless, all chambo species were significantly differentiated in allelic composition from each other (Table 3.11). When the sequential Bonferroni test was applied (5% level) only one locus between *O. squamipinnis* and *O. karongae,* and between *O. squamipinnis* and *O. lidole,* remained significant. There was no significant differentiation at any loci between *O. karongae* and *O. lidole* when the sequential Bonferroni test was applied (Table II.6, Appendix II).

The farm ponds in which chambo had intentionally been stocked (DMOK and MZKH), generally showed the least genetic differentiation from the wild chambo populations, except for the DWST pond (Table 3.11). The high number of 'unique' alleles (Table 3.5) found in the DWST pond and the high incidence of hybridization (due to the unintentional stocking with hybrid fish) may partly explain why this pond was highly significantly differentiated at many loci from the pure chambo species despite stocking, to some extent, with '*O. karongae*'. The DMSH pond (in which

O. shiranus had been stocked) showed significant genetic differentiation from all wild species, although it was slightly less differentiated from *O. shiranus* than from the chambo species. Across all loci, the DWSE pond showed highly significant differentiation from all wild species. However, in the comparison with *O. shiranus,* the DWSE pond differed significantly in allelic composition at only one locus (*IDDH**) (Table II.6, Appendix II).

In all pairwise comparisons of farm pond samples, except one, highly significant differences in allelic composition were observed. Since the founding of farm stocks is likely to be a rather random process (together with the effects of artificial selection to pond environments), a greater level of divergence between farmed compared to the wild populations of the species stocked was perhaps expected. Furthermore, differences occur between farm pond populations in the species intentionally stocked (either *O. shiranus* or chambo), the source of fish, the size of the 'founder population', and the incidence of hybridization. It was therefore surprising that samples from the Domasi *O. karongae* pond (DMOK) and the Mzuzu *O. karongae* holding pond (MZKH) did not show any significant differentiation in allelic composition (Table 3.11), especially since these sites are a great distance apart and were probably stocked with fish from different sources. However, the number of hybrids and chambo found in both samples were very similar (Table 3.6). These results were confirmed by Monte Carlo simulation (10,000 replicates) of genotype contingency tables using diagnostic loci only (*ADA*, ALAT*, CK** and *IDDH**) (Table 3.12).

Table 3.12 Probabilities of exceeding original Chi-squared by chance, in a Monte Carlo simulation of pair-wise comparisons between farm pond populations, based on the genotype contingency tables of the four diagnostic loci. (Individuals with alleles unique to farm populations at the diagnostic loci were not used).

	no.	DMSH	DMOK	DWSE	DWST
DMSH	10				
DMOK	8	<0.001			
DWSE	25	0.006	<0.001		
DWST	35	0.029	0.005	<0.001	
MZKH	10	<0.001	0.999	<0.001	<0.001

DMSH, *O. shiranus* pond Domasi; DMOK *O. karongae* pond Domasi; DWSE Sewage pond Dwangwa; DWST Storage tank pond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu.

The nearest neighbour-joining dendrograms constructed from three different distance measures (Table II.7 and II.8, Appendix II), generally showed a very similar pattern (Figure 3.12-3.14). The agreement between dendrograms, in which distances were based either on a measure with no genetic assumptions (Figure 3.12) or on a genetic measure (Figure 3.13 and 3.14), suggests that the correct relationship between species and farm pond populations has been identified.

Chambo species were always grouped together, and clearly separated from *O. shiranus* (complimenting the previous results). All nodes in the consensus dendrograms, except the Reynolds distance between *O. squamipinnis* and *O. karongae* (Figure 3.13), were significant (occurring in over 50% of the dendrograms generated). Incidently, the node that was not significant was the only one of all dendrograms that did not group *O. lidole* and *O. squamipinnis*.

The dendrograms also generally agreed on the positions of the farm ponds sampled, in relation to each other and to the wild species. Following the previous results, DWSE was generally grouped with *O. shiranus*, due to the high introgression of *O. shiranus* in the pond and the low frequency of unique alleles (particularly when compared to the DMSH pond). The DWST pond was generally found in a position between the two groups of species (*O. shiranus* and chambo), due to the relatively proportional contributions of *O. shiranus* and chambo genes to the population and the high number of unique alleles. The position of the DMOK and MZKH ponds varied slightly between dendrograms, although they were always placed closer to the chambo species than the other ponds (as expected from their stocking histories).

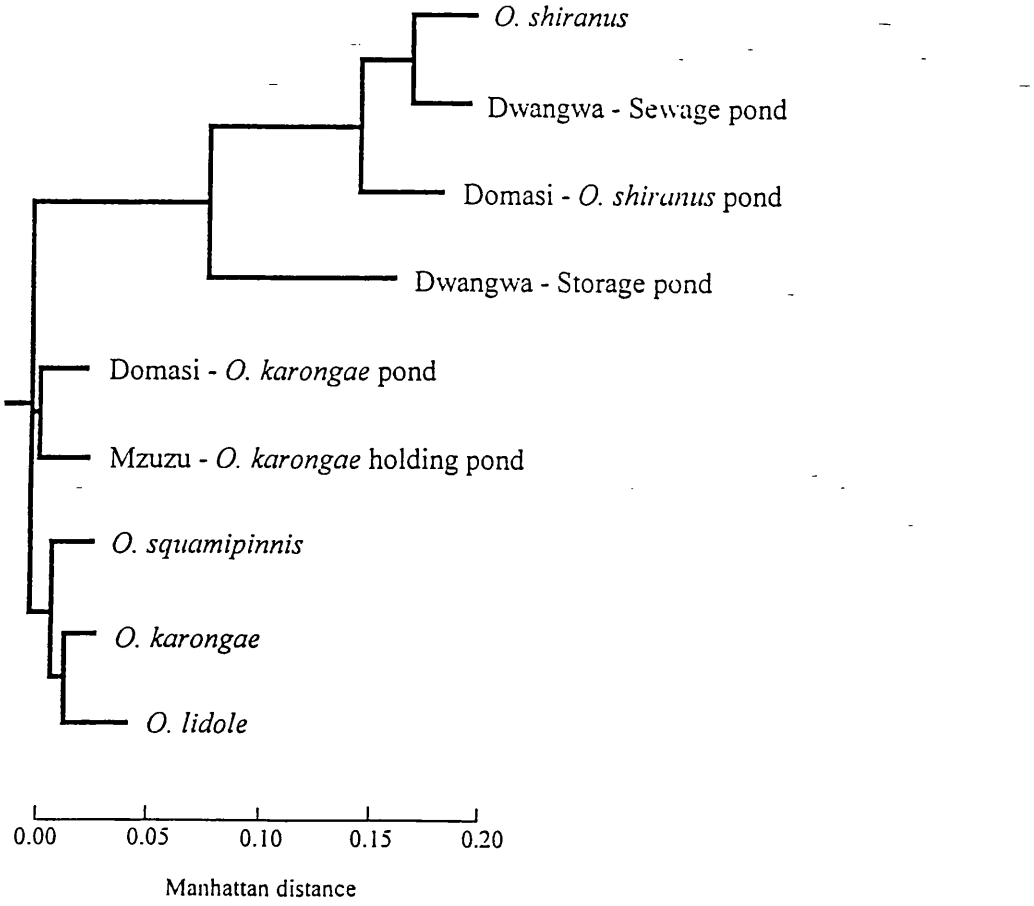


Figure 3.12 Nearest neighbour-joining tree of Manhattan distance, based on allozyme frequencies at 13 loci in samples from wild and farm pond populations in Malawi.

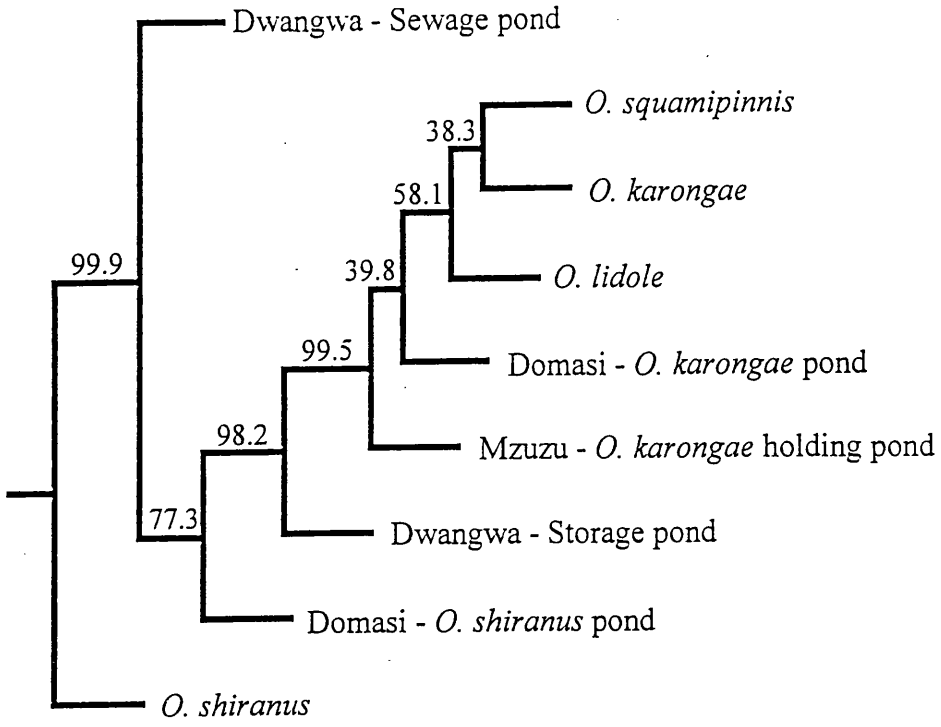


Figure 3.13 Nearest neighbour-joining tree of Reynold's *et al.* (1983) distances, based on allozyme frequencies bootstrapped over 13 loci in samples from wild and farm pond populations in Malawi. Numbers at the forks indicate the percentage of times the group to the right of that fork occurred among the trees, out of 1000 trees.

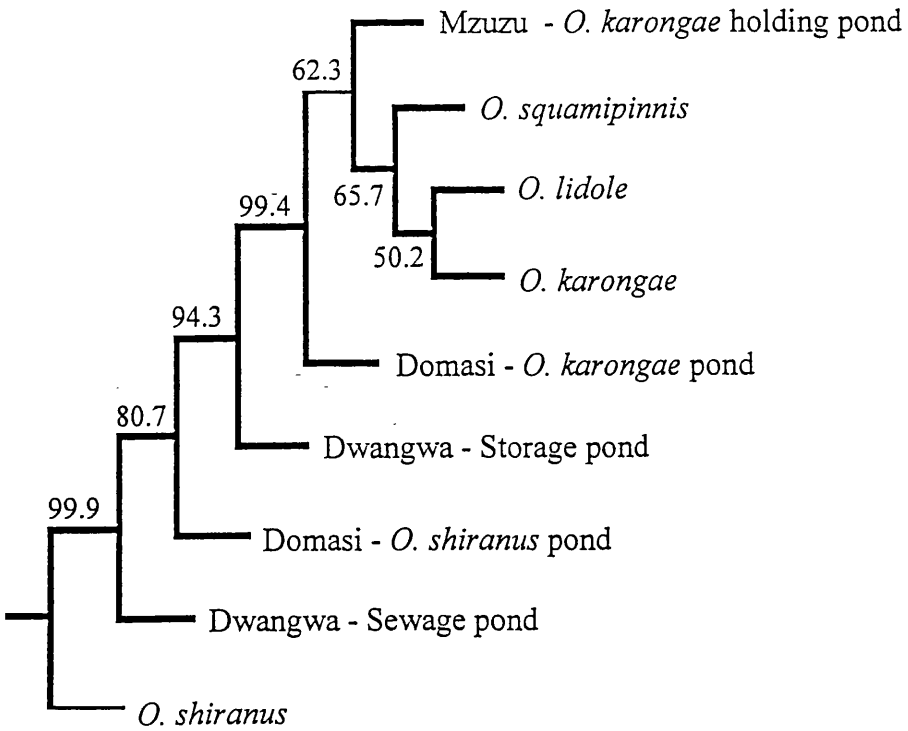


Figure 3.14 Nearest neighbour-joining tree of Cavalli-Sforza's chord measure (1967), based on allozyme frequencies bootstrapped over 13 loci in samples from wild and farm pond populations in Malawi. Numbers at the forks indicate the percentage of times the group to the right of that fork occurred among the trees, out of 1000 trees.

3.4 DISCUSSION

3.4.1 The genetic status of *Oreochromis* species in the wild, Malawi

An understanding of the genetic status of *Oreochromis* species in Lake Malawi, and the surrounding water bodies, is required before the incidence of hybridization in Malawian aquaculture can be fully appreciated. The results from this study, on the genetic status of the chambo species in Lake Malawi, largely agree with the only other genetic study of these species conducted by Sodsuk *et al.* (1995). That is, that the three species are very closely related to each other, with no fixed differences between the chambo species being observed though, unlike Sodsuk *et al.* (1995), very little differentiation in allelic composition was detected. Several alleles were found to be unique to a particular species of chambo, although these largely differed from those observed by Sodsuk *et al.* (1995). Any diagnostic alleles, found in both studies, are therefore unreliable as species markers and a larger study of the chambo species is required.

In this study, the sample sizes of two of the species (*O. lidole* and *O. karongae*) were almost half of those used by Sodsuk *et al.* (1995), and the number of enzyme loci screened was much smaller (13 compared to 43). Despite this, similar F_{ST} values were obtained. The low F_{ST} value of 0.067 ($P<0.005$) is more typical of levels found within a species than between species, and contrasts greatly with the F_{ST} value of 0.527 ($P<0.001$) obtained when *O. shiranus* was included. All chambo species were highly significantly differentiated in allelic composition from *O. shiranus,* and could be clearly separated from *O. shiranus* using four loci.

In contrast to Sodsuk *et al.* (1995) where the lowest expected heterozygosity (H_e) was found in *O. shiranus*, here, *O. shiranus* had the highest value ($H_e = 0.137$). This discrepancy could be due to the prior selection of mainly polymorphic loci (based on the previous study) to increase the information content of loci for the identification of hybrids. Nevertheless, the levels of heterozygosity found in the chambo species compared well to the previous study, ranging from 0.107 to 0.122. These levels are generally higher than those seen in other tilapiines (McAndrew & Majumdar 1983; Taniguchi *et al.* 1985; Rognon *et al.* 1996) and far higher than the mean for all fish (0.051) obtained by Ward *et al.* (1992). Sodsuk *et al.* (1995) suggested that the high heterozygosities observed in the chambo species are the results of the maintenance of original levels of variation, due to a relatively large population size of the different species and the long term stability of the Lake Malawi environment. If population size is an important factor in maintaining levels of heterozygosity, it would be interesting to monitor levels if population sizes continue to decline due to overfishing in future.

The most interesting results obtained from this study are the significant deviations from Hardy-Weinberg equilibrium (HWE) observed in *O. shiranus* and two of the chambo species (*O. karongae* and *O. lidole*). This shows a marked contrast to the study of Sodsuk *et al.* (1995), who reported no such deviations. In the current study, all deviations were due to a deficiency of heterozygotes. Several explanations for such results are possible, of which some are more viable than others. Inbreeding in natural populations is unlikely to be responsible since population sizes are thought to be reasonably large (Turner 1995) and have high levels of genetic variability (as detected in this study). Nevertheless, inbreeding may be possible if populations are subdivided. The incorrect interpretation of gels (i.e. mis-scoring) can result in misleading deviations from HWE, although this is unlikely in this study since all interpretations of zymograms were unequivocal. Significant deficiencies of heterozygotes can be caused by the presence of null alleles, although previous work on these species found no indication to support their existence. Other more viable explanations for the observed deviations from HWE, such as the preferential selection of homozygotes (of interest since only older, sexually mature fish were sampled) and assortative mating, cannot be supported by evidence due to a lack of research in these species.

The reported deviations from HWE could also be due to statistical artifacts, particularly the probability of obtaining a significant result by chance alone. However, tests at individual loci remained significant after the sequential Bonferroni test was applied (Rice 1988), and significant deviations were observed in combinations over all loci. The small sample sizes are also of concern, not only to all statistical tests conducted, but also to the possibility of obtaining a sample unrepresentative of the wild population. However, if this was the case we would perhaps expect to find deviations from HWE due to an excess of heterozygotes, as well as due to a deficiency. Even so, differences in allelic composition between the present study and that by Sodsuk *et al.* (1995), suggests that the samples in both studies were not representative of allele frequencies in chambo populations.

The farm ponds at Dwangwa and Mzuzu are a considerable distance from the sample sites of wild fish taken in this study, and it is known that fish at Dwangwa came directly from the shore of Lake Malawi, only a small distance away. Since wild samples were not collected from this area, and many unique alleles were found in the ponds at Dwangwa, there is further evidence that samples were not representative of the wild populations (if we assume that unique polymorphisms in the farm populations originate from fish in Lake Malawi). Nevertheless, genetic drift in farm pond populations could have significantly increased the frequency of rare alleles in wild

pond populations could have significantly increased the frequency of rare alleles in wild populations. The additional alleles found in this study in both wild and farm populations also suggest that there is genetic variability between sites of *Oreochromis* species in Lake Malawi, and these may represent sub-populations. In fact, the observed deficiency of heterozygotes can also be explained by the subdivision of wild populations (i.e. the Wahlund effect).

Sodsuk *et al.* (1995) were unable to determine the extent of sub-structuring of the various chambo species or *O. shiranus* within the lake, as fish were sampled from only one site (Mangochi area). This site, and the majority of sites in the current study, were situated in the southern arm of Lake Malawi. In the present study, samples of each species were collected from more than one site, so that the sampling of sub-populations could have occurred. The species *O. squamipinnis* was sampled from two different lakes (Lake Malawi and Lake Malombe), although deviations from HWE were not observed. However, only a very small proportion of the total sample (13%) was taken from Lake Malawi. The small sizes of the *O. karongae*, *O. lidole* and *O. shiranus* sub-samples (ranging from 7 to 14) could also explain why no significant genetic differentiation was found between sub-samples (if it is assumed that these sub-samples represent sub-populations). The extremely low F_{ST} values observed within species, the high levels of heterogeneity and the lack of linkage disequilibrium do not support the theory of sub-populations, although again these tests were restricted by the small sample sizes. The observed deficiencies of heterozygotes could be explained by the occurrence of more than one species in the sample, *O. lidole* and *O. karongae* for example, although this is highly unlikely as individuals for each sample were only chosen if they conformed strictly to the characteristics of the type species.

If population subdivision does occur in the chambo species it may be due to the patchy distribution of favourable spawning grounds and feeding sites, which could result in the restricted movement of each species between a specific area. Lowe (1953) described three different nesting sites for each of the chambo species, at different depths and of various types of substrate, which were also thought to act in the reproductive isolation of the these species. However, Turner & Robinson (1991) found no evidence for the strict spatial segregation of breeding grounds, and in one small area (Cape McClear) more than one species was found to be nesting. An extensive overlap in the diet of the three species was also observed. Furthermore, *O. lidole* lives offshore for most of the year and it is the most specialised of the chambo to an open water, plankton feeding existence. Turner *et al.* (1991b) reported that *O. lidole* had a patchier distribution of nesting sites than *O. karongae*, although it was also said that this could be due to difficulty in finding nests in

are thought to migrate 25 km from Lake Malawi to Lake Malombe, via the River Shire, to utilize the lake as a nursery area (Turner & Robinson *unpubl.*). Apart from this, *O. lidole* does not live or breed in Lake Malombe (Tweddle *et al.* 1995). Morphometric analysis of *O. karongae* and *O. squamipinnis* populations, suggest that the stocks of these species in Lake Malombe are distinct from those of Lake Malawi (unpublished data, Tweddle *et al.* 1995). Population subdivision could possibly occur if mature individuals returned to the same spawning ground each season.

O. karongae is the most inshore living of the chambo species, so it is possible that population structuring could occur if movement is restricted. Furthermore, Turner & Robinson (1991) found considerable habitat related geographic variation in morphological traits (particularly of the pharyngeal bone) in *O. karongae*. Geographic variation was also observed in most of the external and pharyngeal measurements of *O. squamipinnis*. However, it is not known whether these varying characteristics have a genetic basis. These observations were also made on a lake-wide level, rather than within just one area of the lake, so they do not provide direct evidence for variation within an area the size of the southern arm of Lake Malawi. Nevertheless, there is strong evidence that populations in the north and the south of the lake are differentiated. A larger study of the *Oreochromis* species in Lake Malawi is required to establish their genetic structure, particularly to ensure the effective management of this important resource.

3.4.2 The genetic status of *Oreochromis* species in farm ponds, Malawi

3.4.2.1 Genetic diversity in farm ponds and differentiation from wild populations

Levels of genetic diversity were higher in all farm ponds sampled than in the wild populations, which initially suggests that farm stocks have not suffered from a loss of genetic diversity. However, since alleles characteristic of more than one species were present in all farm ponds (although 'pure' fish of only *O. shiranus* or chambo were found in each), it is not surprising that levels of genetic diversity are comparatively high. It is impossible to say, unless pure fish are examined separately, whether genetic diversity of an individual species has altered in the farm ponds as compared to the wild populations. A decline in the genetic diversity of farm populations has frequently been reported in the aquaculture of tilapia, due to factors such as a small founder population leading to inbred stocks (Pullin & Capilli 1990; Eknath *et al.* 1991). A number of alleles detected in wild populations were not found in individual pond samples, although seven alleles were detected only in farm pond samples. Apart from inadequate sampling of the wild species (as previously discussed), there may be other reasons for the occurrence of these 'unique' alleles.

Alleles which are not maintained in wild populations, may continue to exist in farm populations if the selection pressures which operate in the wild are removed or relaxed in the pond environment (assuming that these alleles originate from wild fish). Furthermore, if a rare allele is included by chance in the sample used to stock ponds, its frequency in the farm pond would be much higher than in the wild population due to random genetic drift arising from low effective population sizes and / or a founder event (Nei *et al.* 1975). However, the most likely indicator of founder events is, in fact, the loss of rare alleles. An alternative explanation, for the occurrence of the alleles, is based on the observations of rare alleles in the hybrid zones of a number of other species (Clarke 1968; Barton *et al.* 1983). The occurrence of this extra variation has been explained by intragenic recombination in hybrids or due to elevated mutation rates. Since hybrids were detected in all farm ponds the previous explanations are plausible, although the occurrence of these unique alleles in some of the farm ponds were far from 'rare'. New selection pressures in the pond environment, in combination with drift, can bring about marked changes in previously rare alleles.

Some of the alleles restricted to farm populations, have also been observed in other species of tilapia not found in Lake Malawi (Sodsuk *et al.* 1995). It is unlikely that non-endemic species have been introduced to ponds at Dwangwa, since fish were taken directly from the lake. It is however, possible that other species of *Oreochromis* have been introduced to the experimental farm at Domasi. In fact, some fish in the Domasi pond samples had colouration and shape that resembled the species *Oreochromis mossambicus* or *Oreochromis aureus* (E. Roderick, *pers. obs.*). A pilot study into the culture of *O. mossambicus* in community owned rainfed pools in the Zomba area, has recently been published (Chikafumbwa *et al.* 1997). It is therefore likely that *O. mossambicus* is also present in the farm ponds at Domasi. The Malawian Government has prohibited the use of any other non-endemic species.

Due to the occurrence of these unique alleles in ponds and / or the occurrence of alleles diagnostic of both species (i.e. *O. shiranus* and chambo), significant genetic differentiation was observed between all ponds and wild populations. However, the chambo species were least different from ponds in which pure chambo were intentionally stocked (DMOK and MZKH). Pond populations (except between DMOK and MZKH) also differed significantly in genetic composition, also presumably as a result of the presence of different alleles and the varying proportions of *O. shiranus* and chambo alleles due to their stocking histories.

Significant deviations from Hardy-Weinberg, due to a deficiency of heterozygotes, were detected in three of the five ponds examined. As with the wild populations, there are several

explanations for this observation. For example, genetic drift may result in an excess of homozygotes, as is commonly observed in inbreeding farm pond populations (Kincaid 1983; Taniguchi *et al.* 1985; Hulata *et al.* 1988). The Wahlund effect, due to the presence of more than one species (or population), may explain the observed deviations from HWE. Furthermore, hybridization between species or populations can result in a deficiency (or excess) of heterozygotes. Pure individuals of only chambo or *O. shiranus* were observed in pond populations, though genetic substructuring may occur between hybrids and pure species, and within species of chambo. It is, in fact, highly likely that more than one species of chambo is present within the ponds, since these are far harder to separate than *O. shiranus* is from chambo. However, as will be discussed further, it is likely that chambo species also interbreed in captivity. The addition of fish to the pond, such as the annual restocking of ponds at Dwangwa, may also produce genetic substructuring within the pond population. If a population remains isolated, and is freely intermixing, with time it may reach equilibrium. These circumstances may explain why no significant deviations from HWE where observed in the DMSH and DMOK ponds.

3.4.2.2 The incidence of hybridization

Hybrids, between *O. shiranus* and chambo, were found in every farm pond sampled in Malawi. No F_1 hybrids were found, which indicates two important points. Firstly, hybrids are fertile and, secondly, the detection of post F_1 hybrids is strongly suggestive that hybridization may be introgressive. The higher levels of genetic variability observed, resulting from the creation of new genotypes through genetic recombination, are also a characteristic of introgressed populations. The incidence of hybridization was largely related to whether any preference for one species (either chambo species or *O. shiranus*) took place during stocking. For example, the greatest number of hybrids were found in the ponds at Dwangwa where the species of fish stocked was largely indiscriminate. Whereas, the MZKH pond at Mzuzu, which was well managed and intentionally stocked with *O. karongae*, contained relatively few hybrids

Out of both ponds at Dwangwa, more hybrids were detected in the DWST even though chambo (*O. karongae*) were preferentially stocked. However, the fish used to stock this pond were largely taken from the DWSE pond where the chambo-type fish were unlikely to be pure. Furthermore, stocking of this pond took place only relatively recently (1994), whereas the DWSE has existed since at least 1978. It is therefore, highly likely that a great deal of introgression has taken place within the DWSE pond, and the 'pure' *O. shiranus* genotypes detected (based on four

diagnostic loci) are in fact hybrids. These circumstances may also explain why all loci in the DWSE sample were in linkage equilibrium, whereas significant linkage disequilibrium was detected in the DWST pond sample. The interpretation of stocking history related to the incidence of hybridization is further complicated by the annual restocking of these ponds.

The high number of hybrids found in the DMSH pond at Domasi, was surprising since only *O. shiranus* should have been stocked. However, it was apparent when sampling, that other species of *Oreochromis* were also present in the pond. Several factors may explain why more than one species, and therefore hybrids, were found in all farm ponds which were originally meant to contain one species only. Firstly, the accidental stocking of more than one species, particularly if fish were introduced as fry when species identification is very difficult. However, it is relatively easy to separate chambo from *O. shiranus* as adults and mixing of fish is therefore more likely to have occurred after ponds were stocked with one species. Transfer of fish between ponds is highly likely during the rainy season when the ponds flood. For example, in April 1995 many ponds at Mzuzu became united when severe flooding brought the level of water in ponds over the height of the subdividing banks. Furthermore, otters have been a serious pest at Mzuzu and it is possibly that these may have, albeit in low frequencies, transferred fish between ponds. In fact, other piscivorous animals, such as fish eagles, have been blamed for dropping fish into ponds containing a different species (Elder *et al.* 1971). Elder *et al.* (1971) further suggested that species-contamination of *Tilapia spilurus nigra* ponds with *Tilapia zillii* occurred via inlet pipes during the filling or topping up of *T. s. nigra* ponds. Other transfers of fish, due to human error, may occur if fish are thrown back into the wrong ponds after sampling.

3.4.2.3 Other factors influencing the incidence and nature of hybridization

The degree to which *O. shiranus* and chambo have been physically separated (prior to or after stocking) has a large influence on the incidence of hybridization. Furthermore, the dominance of one species, due to its preferential stocking, also affects the nature of hybridization. For example, the DMSH pond was intentionally stocked with *O. shiranus*, and indeed the hybrid genotypes were dominated by *O. shiranus* alleles (i.e. progeny of hybrid back-crossed with *O. shiranus*). A relative scarcity of conspecific mates (the 'Hubbs principle', Hubbs 1995) may explain the hybridization of the rarer species. F_1 hybrids are in fact more likely to mate with the type of fish which is most common.

The frequency of back-crossing may be more common if hybrids suffer reduced

reproductive competence, either in terms of mating behaviour or gamete quality and quantity (Verspoor & Hammer 1991), though, the relative fitness of chambo x *O. shiranus* hybrids is unknown. Hybrids between other species of tilapia vary in fitness depending on the species crossed. Great attention has been paid to the species which produce hybrid progeny with increased fitness traits (heterosis), such as growth performance, and especially to those which produce largely all-male progeny (Schwartz 1983; Wohlfarth 1994).

The direction of hybridization may also be effected by how 'choosy' a particular species is. For example, if mating was completely random in the DWSE pond (where no species-specific stocking occurred) and if we assume that equal numbers of each species entered the pond, then we would expect to find an approximately equal frequency of *O. shiranus* and chambo alleles in the hybrids. However, the sample was dominated by *O. shiranus* alleles. If *O. shiranus* was not dominant in the founder fish, such asymmetric mating may be due to a less discriminatory choice in the species of mate by *O. shiranus*. An alternative explanation is if *O. shiranus* reproduces at a quicker rate than chambo. Indeed, chambo attain first maturity and reproduce in ponds only after reaching 90 g body weight, whereas *O. shiranus* mature and reproduce at 8 g (above 25°C) (Maluwa & Dickson 1993).

It is likely that other factors, such as stocking densities and water visibility, may influence the incidence of hybridization in farm ponds. All ponds were quite densely populated and excessive breeding was evident due to the large size range of fish. The greatest density of fish and the highest numbers of brooding females were observed in the richly fertilized ponds at Dwangwa, where the incidence of hybridization was in fact also the greatest. At all farms, details such as the stocking density and sex ratios of fish in ponds were rarely available, so no clear conclusions can been drawn. Nevertheless, it is apparent that where the greatest efforts had been made to control the number of fish in the pond and keep chambo and *O. shiranus* separate, the incidence of hybridization is lower.

3.4.2.4 Causes of hybridization

It is evident from this study that when *O. shiranus* and chambo are found within the same pond they will hybridize. There are no reports to suggest that hybridization between these species occurs in the wild (Turner *et al.* 1991b), therefore conditions within the pond allow the natural pre-zygotic isolating barriers to breakdown. Reproductive isolation may be prevented for the following reasons:

1) Lack of habitat separation - The limited variation in water depth (generally no deeper than 1 m) and continuous form of substrate, do not allow the nesting habitats of chambo and *O. shiranus* to be separated as they would be in the wild. *O. shiranus* generally breeds in the vegetated areas of shallow waters (to a depth of 4 m) (Trewavas 1983). The breeding sites of chambo greatly overlap, occurring at a variety of depths (from 0.5 m to at least 28 m in *O. karongae*, from less than 4 m up to 20 m in *O. squamipinnis*, and from 17 m to at least 50 m in *O. lidole*). All chambo species breed on a variety of substrata, including sandy, muddy and rocky areas (Turner & Robinson 1991; Turner *et al.* 1991b). Limited nesting space due to far higher population densities than would be observed naturally, also mean that nests are closer than would be expected in the wild. Chambo males tend to nest well apart from conspecifics in the wild, where interactions between neighbouring males are extremely rare. In farm ponds, where nesting areas are crowded, opportunities for 'sneaky matings', such as those documented between the brown trout (*Salmo trutta*) and Atlantic salmon (*S. salar)* (Crozier 1984), may occur.

2) Lack of temporal isolation - The continuous supply of food or reduction in the impact of seasonal cues in captivity may prevent the species being temporally isolated. However, in the wild the main breeding season of *O. shiranus* (September to March) does overlap with that of chambo (all species at some time between August and March), being prompted to some extent by the beginning of the rainy season (Trewavas 1983; Turner & Robinson 1991; Turner *et al.* 1991b). Nevertheless, in captivity, *O. shiranus* can breed all year and females are capable of spawning at 4 - 8 week intervals (Mattson & Kaunda 1997). Fish may also mature earlier in the ponds and therefore have a greater reproductive lifespan.

3) Inability to identify conspecifics - Visibility is far poorer in farm ponds (21.0 - 60.5 cm, in this study) than in the wild (3 - 20 metres; Konings 1990). The reduced clarity of pond water may prevent individuals from being able to accurately identify conspecifics due to a lack of contrast in colour (Seehausen *et al.* 1997; Seehausen & van Alpen 1998) and definition of movement during courtship displays. Once hybrids are generated the species-specific behavioural and morphological characteristics, which maintain reproductive isolation, become less defined making the identification of conspecifics more difficult.

4) Scarcity of conspecifics - If conspecifics are rare (due to stocking), individuals may have little option but to mate with a non-conspecific (e.g. observed in sunfish, *Lepomis* species (Avise & Saunders 1984), and in cichlids (Crapon de Caprona 1986)).

Since isolating barriers break down between *O. shiranus* and chambo, which are relatively

highly differentiated, then it is highly likely that reproductive isolation is not maintained between the very closely related chambo species. There is no evidence to suggest that these species hybridize in the wild. Female choice for chemical or behavioural cues are thought to be extremely important in maintaining the reproductive isolation of these species (Turner *et al.* 1991b). Poor visibility and the high density of nests in farm ponds may reduce the effectiveness of behavioural and chemical cues, respectively. Hybridization between species of chambo, firstly requires that more than one species is present in the farm pond. This is highly likely, especially when fish were stocked indiscriminately in one pond, and because the three species of chambo are far harder to discriminate than are *O. shiranus* and chambo. Furthermore, chambo fingerlings caught by beach seining have been reported to be used in the stocking of farm ponds (Maluwa *et al.* 1995), and it is impossible to separate the three species at this young age. Chambo species-specific diagnostic markers would be highly valuable in assessing the extent of hybridization.

3.4.3 The consequences of hybridization to farm and wild populations

Tilapia are well known to have a strong propensity to hybridize in captivity and in the wild, so the findings of this study are not surprising. The frequent decline of performance in tilapia, due to unintentional introgressive hybridization, has underlined the importance of ascertaining the identity of the species cultured (Taniguchi *et al.* 1985; Macaranas *et al.* 1986). Hybridization has frequently been reported in wild populations of tilapia when originally allopatric species have come into contact after transplantation (Fryer & Iles 1972; Daget & Moreau 1981; Ogutu-Ohwayo & Hecky 1991). The widespread escape of hybrids from farm ponds into the wild could also have serious evolutionary consequences for all the *Oreochromis* species of Lake Malawi. Genetic introgression of non-native species into natural populations has had adverse effects on some species leading to a decline of native populations, as seen after the introduction of brook trout into native bull trout populations (Leary *et al.* 1993), and has often led to the elimination of one of the parental species in tilapia populations (Moreau 1983). The introduction of escaped farm hybrids into Lake Malawi may have a similar effect. Avenues for escape were apparent at all farms, and were likely to increase during the rainy season. In fact, flooding of smallholder fish ponds is reported to be one of the major risk factors to aquaculture resulting in the complete renewal of stocks (Chimatiro & Janke 1994). Furthermore, farm fish have intentionally been released into other small water bodies such as reservoirs and other farm ponds, and fish have even been used to restock Lake Chilwa.

The ecological requirements of the chambo species are very similar. They are believed to form an ecological series, showing increasing specialisation to an open-water, plankton feeding existence (Lowe 1953; Robinson & Turner 1990). However, it is not completely certain what factors maintain reproductive isolation between these endemic species (Turner *et. al.* 1991a, b). It is therefore, highly likely that these species are particularly susceptible to hybridization if factors which maintain pre-zygotic isolation are disturbed through the introduction of hybrids into Lake Malawi. Hybrids could form a route for a breakdown in pre-zygotic isolation barriers which exists in all *Oreochromis* species at present, and lead to the consequential loss of co-adapted gene complexes which allow for niche separation and its associated population and species biodiversity. A further threat to the endemic species could be due to the hybrid vigour and enhanced reproductive potential often exhibited in interspecific hybrids. Farmed fish, whether they are hybrids or not, may have diverged from their wild conspecifics and further threaten the locally adapted populations. The detrimental effects of interbreeding and competition between farmed and native fish have been extensively documented in the Atlantic salmon, *Salmo salar* (Einum & Fleming 1997). Hybrid and farmed salmon are more aggressive, dominating the native fish in contests and habitat use. Cultured fish therefore pose a serious threat to the unique tilapiine species flock and also to the *Oreochromis* fishery of Lake Malawi.

3.5 SUMMARY

The findings of this study can be summarised as follows:

1) Allozyme analysis detected no fixed genetic differences between the endemic *Oreochromis (Nyasalapia)* (chambo) of Lake Malawi. A larger study, possibly using a more sensitive molecular technique, is required to confirm if any diagnostic markers of these species exist. All chambo species exhibit high levels of genetic diversity, are highly differentiated in allelic composition from *O. shiranus*, and can be clearly separated from the latter species using diagnostic loci.

2) A broader study of the *Oreochromis* species in Lake Malawi is required to confirm their genetic structure, to ensure the effective management of this important resource which is threatened by overfishing.

3) Levels of genetic diversity higher than those of the wild populations were detected in all farm ponds sampled, mainly due to the presence of more than one species. In general, farm pond populations were significantly differentiated from the wild populations sampled. The observed genetic differentiation is thought to be derived from a number of factors including hybridization,

genetic drift, the inadequate sampling of the wild populations and artificial selection to pond environments. The previous factors, as well as different stocking histories (including the species intentionally stocked, the source and size of the founder population), may also explain why high levels of differentiation were found between farm pond populations.

4) Hybridization between *O. shiranus* and chambo was observed in every farm pond sampled. No F_1 hybrids were detected, indicating that hybrids are fertile. Evidence of extensive introgressive hybridization was found in some farm ponds based on the high incidence of back-crossed hybrid individuals and their apparent reproductive competence. The incidence of hybridization is largely dictated by the degree to which stocking has been species-specific.

5) A number of factors are likely to contribute to the breakdown of reproductive isolation between *O. shiranus* and chambo in farm ponds, including a lack of habitat separation, poor water visibility, the crowding of nesting areas, and a scarcity of conspecific mates. It is highly likely that hybridization also occurs between the chambo species in aquaculture.

6) The introduction of cultured fish, via escape from farms, poses a serious threat to the unique tilapiine species flock and also to the *Oreochromis* fishery of Lake Malawi, through predicted effects on the maintenance of reproductive isolation between species and on the unique coadapted gene complexes of species.

CHAPTER 4
HYBRIDIZATION IN FARM PONDS, MALAWI: EVIDENCE FROM RAPD

4.1 INTRODUCTION

In the previous chapter, allozyme data provided evidence for the breakdown in reproductive isolating barriers between *O. shiranus* and chambo (*Oreochromis (Nyasalapia)* of Lake Malawi) in farm ponds of Malawi. It was suggested that since *O. shiranus* and chambo are relatively highly differentiated, then it is likely that in captivity reproductive isolation is not maintained between the very closely related chambo species. However, allozyme electrophoresis was not powerful enough to discriminate between the three chambo species. For this reason it was also not possible to determine whether one particular chambo species was hybridizing more frequently with *O. shiranus* in farm ponds. Chambo species-specific markers would be highly valuable in assessing the characteristics and extent of hybridization.

Random amplified polymorphic DNA (RAPD) analysis (Williams *et al.* 1990; Welsh & McClelland 1994), in which a single primer of short (10-15 bp) arbitrary DNA sequence is used to amplify random DNA segments, has proven to be a valuable tool in the generation of species-specific markers (e.g. in mosquito species (Kambhampati *et al.* 1992), and fish species (Dinesh *et al.* 1993)). RAPD markers that discriminate among closely related taxa have been useful for assessing and quantifying the extent of introgression and hybridization among species (e.g. Arnold *et al.* 1991; Khasa & Dancik 1996). RAPD primers consist of random sequences which do not discriminate between coding and non-coding regions and thereby provide an opportunity to sample far more loci than conventional methods such as allozyme electrophoresis (Lynch & Milligan 1994). This technique has therefore been used for the analyses of hybridization events where allozyme studies have not proven to be sensitive enough to identify hybrid genotypes (e.g. *Brassica* species (Quiros *et al.* 1991)). The large amounts of genetic variation revealed by RAPDs (Hadrys *et al.* 1992) have also been used to improve discrimination within and between populations and strains (e.g. conspecific populations of the medfly, *Ceratitis capitata* (Baruffi *et al.* 1995) and the red-cockaded woodpecker, *Picoides borealis* (Haig *et al.* 1996)). The RAPD technique is efficient and cost-effective, there is no need for radiolabelled probes, and the use of random primers avoids the need to obtain sequence data for the design of species-specific primers. However, there has been some concern over the reliability and reproducibility of RAPD generated

banding patterns (Lynch & Milligan 1994; Van de Zande *et al.* 1995; Jones *et al.* 1996).

Bardakci & Skibinski (1994) used RAPD markers to discriminate between three species of tilapia (*Oreochromis aureus*, *O. niloticus* and *O. mossambicus*) and also between several subspecies of *O. niloticus*. RAPD analysis was more successful than previous studies using allozyme analysis (Seyoum 1990) in distinguishing among subspecies of *O. niloticus* (Bardakci & Skibinski 1994). RAPD has also been used to evaluate genetic diversity between aquacultural strains of *O. niloticus* from the Philippines (Naish *et al.* 1995b). Bardakci (1996) developed three primers of sequence-characterized amplified regions (SCARs), from the products of RAPD analysis, that revealed some length variations between different species of tilapia (but not between strains). The primer OPB 06_{374} allowed three species (*O. mossambicus*, *O. aureus* and *Tilapia mariae*) of tilapia to be identified clearly, although no length variation was detected between the SCAR products of *O. spilurus* and *O. niloticus*. Single-strand conformation polymorphism (SSCP) analysis of the SCAR products of OPB 06_{374} clearly distinguished homozygous and heterozygous individuals. In a cross between *O. niloticus* and *O. mossambicus*, the parents had single bands (homozygous) of different lengths and their offspring had both fragments (heterozygous). SSCP analysis, which allows the detection of single-base changes in short DNA fragments, may therefore be highly valuable in distinguishing chambo species, and the extent of hybridization among them and between *O. shiranus* in farm ponds.

The aims of this study, using RAPD analysis, were:

1) To provide an appraisal of the RAPD technique - by examining its reproducibility, reliability, and its analytical power, through a preliminary study of wild and inbred lines of tilapia as well as through the study of the *Oreochromis* species of Malawi.

2) To identify diagnostic marker fragments of the three chambo species and *O. shiranus*, by RAPD, SCAR and /or SSCP analyses, so that the incidence of hybridization can be assessed not only between chambo and *O. shiranus* but also among the chambo species.

3) To compare RAPD and allozyme analysis based estimations of the incidence of hybridization in farm ponds and of the genetic relationships among chambo, *O. shiranus*, and farm pond fish.

4.2 MATERIALS AND METHOD

4.2.1 Preliminary investigation

To investigate the analytical power and reliability of the RAPD technique, a preliminary investigation was undertaken in which RAPD was used to detect any loss of variation which may

be caused by inbreeding within lines of tilapia. Cultured fish may become inbred due to the continuous use of one stock and progressive loss of heterozygosity (Kincaid 1983). It is valuable to detect inbreeding which may be detrimental if deleterious genes are expressed in the homozygous state, and if variation is lost. DNA was analysed from the fish of wild (1 male and 1 female) and inbred lines (F_2 and F_3 generations; 1 male and 5 females of each generation) of *Oreochromis spilurus*. The F_2 and F_3 generations of *O. spilurus* had been bred in captivity by Eric Roderick at the University of Wales Swansea. An unknown line of *O. spilurus* from Stirling University (1 male and 1 female) was also analysed to establish if it was possible to assess the likelihood of inbreeding in this line.

The extraction of DNA and RAPD protocols are described in sections 4.2.3 and 4.2.4, respectively. Fifteen random decamer primers (Operon Technologies Inc.) of divergent sequences (to ensure that different parts of the genome were analysed) were screened, of which nine produced repeatable banding patterns of high resolution which were used for further analyses (see Table 4.1). The consistency and reproducibility of amplification products was tested by amplifying three of the samples, three times and visually comparing the banding patterns. The consistency of scoring was also tested by scoring the same gel three times. The method of scoring and analysis are described in sections 4.2.5 and 4.2.8, respectively.

4.2.2 Sampling

Details of sample sites and the number of caudal fin tissue samples collected are given in Chapter 3 (Figure 3.5 and Table 3.2). Unlike allozyme analysis, the collection of tissue for RAPD analysis did not require the sacrifice of a fish and therefore allowed the collection of a greater number of samples. For this reason, samples for RAPD analysis only were taken from the garden pond at Chirombo Bay, the *O. shiranus* holding pond (MZSH) and *O. karongae* breeding pond (MZKB) at Mzuzu. Fin tissue samples of 278 fish were collected in total (Table 3.2), of which 185 samples provided useable DNA (Section 4.3.2). Seven individuals of two other species in the genus *Oreochromis* (*O. aureus* and *O. mossambicus),* which had successfully been analysed in previous RAPD studies (University of Wales Swansea), were also analysed to represent reference species.

4.2.3 DNA extraction

DNA was prepared from fin tissue following the method described by Bardakci & Skibinski (1994) with some modifications. The fin tissue samples taken from fish kept at the University of

Swansea was processed immediately, whereas tissues collected in Malawi and stored in 20% DMSO salt saturated solution were blotted carefully to remove as much preservation solution as possible. Approximately 100 mg of tissue was suspended in 500 μl STE (0.1 M NaCl, 0.05 M Tris and 0.01 M EDTA, pH 8.0) and cut into small pieces. After adding 40 μl SDS (10%) and 40 μl Proteinase K (Boehringer, 10 mg / ml) the suspension was gently mixed and incubated at 50°C for 30-40 minutes. DNA was purified by three successive extraction stages using equal volumes of phenol, phenol:chloroform:isoamylalcohol (25:24:1) and then chloroform: isoamylalcohol (24:1). At each stage the mixture was mixed on a rotator for 10 minutes, and centrifuged for 2 minutes to separate the organic (phenol, chloroform) from the aqueous phase (DNA, buffer). To avoid shearing of the DNA, a wide bore pipette tip was used to transfer the aqueous phase to a new tube.

DNA was precipitated by adding twice the volume of ice-cold absolute ethanol, and centrifuged for 3 minutes before decanting off the liquid. The pellet was washed with 70% ethanol for 1 hour to dissolve all remaining salts. After drying at 37°C for 10 minutes, the DNA pellet was suspended in 150 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and stored at 4°C. The quality of the DNA was checked on 1% agarose gels with ethidium bromide staining, and quantified using a GeneQuant (Pharmacia). Many (33.4%) of the tissue samples collected in Malawi only provided degraded non-amplifiable DNA (Section 4.3.2). These were double checked by another extraction from the original tissue, although usually without any improvement in DNA quality.

4.2.4 RAPD protocol

A set of 28 random decamer primers (Operon Technologies Inc.), of disparate sequences, were used initially to screen for species-specific fragments of the four species sampled from Malawi (Table 4.1). The primers were selected since they had produced the clearest and most informative banding patterns in a recent tilapia mapping project at the University of Wales Swansea (K. Naish *pers. comm.*). From the initial screening, four primers producing the clearest and most reliable species-specific fragments (Table 4.1) were chosen for the further analysis of wild and farm pond *Oreochromis* populations in Malawi. The amplification conditions were based on Williams *et al.* (1990) and Bardakci & Skibinski (1994) with some modifications.

The PCR reaction contained 2.5 μl of reaction buffer, 2.0 mM of $MgCl_2$, 100 μM dATP, dTTP, dGTP and dCTP (Pharmacia), 0.5 μM primer, 0.5 units *Taq* polymerase ('Red Hot' polymerase; Applied Biotechnologies) and 20 ng of template DNA in a final volume of 25 μl (using sterile, filtered water). DNA pooling can be an effective strategy for detecting genetic

differences between species and can increase efficiency by decreasing the need to analyse all individuals (Williams *et al.* 1993; Michelmore *et al.* 1991; Furman *et al.* 1997). Therefore, 100 ng of genomic DNA from four to twelve individuals of each species (depending on the number of individuals available) was pooled and 20 ng of this pooled sample used as template DNA for the reaction mixture. However, the reproducibility of banding patterns was not satisfactory (see section 4.3.1.2) so this approach of pooling DNA was discontinued, and all individuals were analysed separately.

Reactions were overlaid with 25 μl of mineral oil (Sigma) to avoid evaporation and PCR performed in an Omnigene Thermocycler (Hybaid, U.K.) with the temperature cycle shown in Table 4.2. After completion of the PCR, 5 μl of sucrose loading buffer was added to the amplification product to monitor the progress of the electrophoresis. 15 μl of this mixture was separated on a 6% vertical nondenaturing polyacrylamide gel in TBE buffer (0.89 M Tris, 0.89 M Boric acid and 0.11 M EDTA, pH 8.3). Polyacrylamide gel electrophoresis increases the resolution of band (i.e. fragment) separation compared to agarose gel electrophoresis. Fourteen samples were run at a time with Boehringer marker VI in one lane as a DNA size marker. A negative control, which consisted of all reaction ingredients except the DNA template, was used to detect

Table 4.1 Sequence and Operon codes of the random primers used in the RAPD analysis of *Oreochromis* species.

Primer codes	Sequence (5' to 3')
OPA 08†*	GTGACGTAGG
OPA 10*	GTGATCGCAG
OPB 06†	TGCTCTGCCC
OPB 07†	GGTGACGCAG
OPA 11†	CAATCGCCHT
OPB 12†	CCTTGACGCA
OPC 01†	TTCGAGCCAG
OPC 02†	GTGAGGCGTC
OPC 05†	GATGACCGCC
OPC 10†	TGTCTGGGTG
OPC 11	AAAGCTGCGG
OPD 02	GGACCCAACC
OPD 03	GTCGCCGTCA
OPD 11	AGCGCCATTG
OPD 15	CATCCGTGCT
OPE 11	GAGTCTCAGG
OPE 15*	ACGCACAACC
OPE 17	CTACTGCCGT
OPF 11	TTGGTACCCC
OPF 14	TGCTGCAGGT
OPF 20	GGTCTAGAGG
OPG 02	GGCACTGAGG
OPG 06	GTGCCTAACC
OPG 10	AGGGCCGTCT
OPG 12	CAGCTCACGA
OPG 17*	ACGACCGACA
OPG 18	GGCTCATGTG
OPG 19	GTCAGGGCAA

† Primers used in the preliminary investigation (see section 4.2.1).
* Primers selected for further analyses of wild and farm pond populations of *Oreochromis* species from Malawi.

contamination. No contamination was observed in any reaction. Gels were run at 30 mA for 3.5 to 4.5 hours depending on the size of fragment of interest. Long running times during electrophoresis greatly reduces the probability of scoring non-homologous fragments as homologous. Standard silver staining procedures were employed to visualise the fragments (Table 4.3). After staining, to prolong the resolution of the bands, the gel was rinsed in solution A once again (containing twice the original amount of 1% Acetic acid) and then sealed in a plastic bag.

Table 4.2 PCR cycle program for RAPD

Temp. (°C)	Time (s)	Function
1st Cycle:		
94	120	Initial denaturation
35	60	Annealing of primers
72	120	Extension
44 Cycles:		
94	30	Denaturation
35	60	Annealing
72	120	Extension

Table 4.3 Silver Staining Procedure

Step	Solution	Details
Fix	A: 10% Ethanol, 0.5% Acetic acid	Twice, for 3 min.
Stain	B: 0.1% Silver nitrate solution	10 min.
Rinse	Distilled water	Twice
Develop	C: 1.5% NaOH, 0.1% $NaBH_4$, 0.15% CH_20	Until bands appear

4.2.5 Scoring

The mobility of the amplified fragments from the origin was measured to the nearest 0.5 mm directly from the gel over a light-box. The measurements were converted to base-pair lengths using the computer program of Schaffer & Sederoff (1981). It was assumed that the RAPD fragments amplified from a specific primer and that have the same mobility (confirmed by comparisons across gels by eye and by fragment size) are homologous, and that fragments from different loci do not co-migrate to the same position on a gel. A further assumption was that each fragment ('locus') could be treated as a two allele system, with only one of the alleles per 'locus' being amplified by the PCR reaction.

The gels were first examined for fragments that were unique and conserved among all individuals of each of the four species (*O. shiranus* and the three chambo species: *O. karongae*, *O. lidole* and *O. squamipinnis*), and could therefore be used to disclose the identity of farm pond fish (pure species and hybrids). Since species-specific DNA fragments were not detected for individual chambo species, the gels were examined more closely and a greater number of fragments scored. Only RAPD fragments that were amplified intensely, of clear resolution and polymorphic (i.e. not present in all fish) were used. A minimum of two chambo species and two

O. shiranus were always run on the same gel as the farm pond fish, which confirmed the identity of the species-specific fragments and allowed the consistency of amplification products to be checked. The RAPD fragments chosen for analyses were scored as present or absent and were assigned a value of 1 and 0, respectively.

4.2.6 SCAR (Sequenced Characterized Amplified Regions)

The protocol and primers developed for SCAR analyses are those of Bardakci (1996), used to discriminate between five species of tilapia. In this study, two individuals of each species of chambo and three individuals of *O. shiranus* were used to represent the four species concerned, with a single *Oreochromis aureus* as a control. The control sample had previously been successfully amplified by F. Bardakci, using SCAR primers. The sequences of the three SCAR primers used in this study are shown in Table 4.4, together with the PCR cycle details. The PCR reaction contained 2.5 μl of reaction buffer (Promega), 1.5 mM of $MgCl_2$, 100 μM of each dNTP, 0.5 μM of primer, 0.5 units of *Taq* polymerase (Promega), and 20 ng of template DNA in a final volume of 25 μl . Reactions were overlaid with 25 μl of mineral oil, and amplification preformed in an Omnigene Thermocycler. Various changes in annealing temperature (decreased by 5°C) and magnesium chloride concentration (increased by 0.5 mM) were made if the product was not satisfactory. Amplification products (5 μl) were separated on 1.5% agarose gels and 6% polyacrylamide gels in TBE buffer and visualised with ethidium bromide staining and silver nitrate staining, respectively.

Table 4.4 SCAR primer sequences and PCR cycle programs (Bardakci 1996). For the first cycle, all primers had a denaturation of 94°C for 120 s and extension of 72°C for 90 s. Annealing times and temperatures varied between primers (details shown below). For all primers, the denaturation time was decreased to 30 seconds for the following 33 cycles.

Primer	Sequence (5' to 3')	Annealing details
OPA 13 $_{590}$	CAG CAC CCA CGT GAC TCC CAG CAC CCA CCC AGG TAA A	45 s at 61°C
OPA 13 $_{310}$	CAG CAC CCA CCA ATT ATA ACA AGC ACC CAC TGC ACT ACA AC	120 s at 56°C
OPB 06 $_{374}$	TGC TCT GCC CTG GTG TAG AT TGC TCT GCC CAG ACA TCT C	45 s at 56°C

4.2.7 SSCP (Single Strand Conformational Polymorphism)

SSCP was performed on the PCR products of SCAR primer OPB 06_{374} (the PCR products of primers OPA 13_{590} and OPA 13_{310} were not good enough for SSCP analyses, see section 4.3.4) according to Bardakci (1996). 5 μl of sequencing stop buffer was added to 3 μl of PCR product. The DNA was denatured in a pre-heated block at 95 °C for 10 minutes and then chilled in ice for 30 minutes. The single strands were separated on a 5% polyacrylamide gel in TBE buffer for 2 hours (200 v) at 20 °C using a cooling system, using an undenatured sample as a control. The gels were visualised using the standard silver nitrate staining procedure. The procedure was repeated but with 4 μl of PCR product (6 μl of sequencing stop buffer) and separated on a 10% polyacrylamide gel for 5 hours to increase the clarity of the bands.

4.2.8 Analyses

For the preliminary investigation (section 4.2.1), a mean similarity index (S.I.) was calculated for each primer and across all primers for every group of fish studied, using the formula

$$S_{xy} = 2\, n_{xy} / (n_x + n_y), \qquad \textbf{(4.1)}$$

where n_{xy} is the number of bands that individuals x and y share in common and n_x and n_y are the number of bands scored for each individual (Nei & Li 1985). Shared presence alleles are more likely to be homologous than shared nulls, therefore a similarity index based on the sharing of expressed bands alone should be more robust than one that weighs shared presence alleles equally with shared nulls (Grosberg *et al.* 1996). To determine if the RAPD technique can detect any loss of variation which may be evident in inbred lines as compared to wild lines, Wilcoxon match-pairs signed-rank tests were carried out on the S.I. values between the various groups of *O. spilurus*. To test if the S.I. values for the wild line differ overall from the other lines (F_2, F_3 and Stirling), a combined probability test was carried out using the formula

$$X^2 = -2 \sum ln_e P, \qquad \textbf{(4.2)}$$

where P is the probability value calculated in each Wilcoxon signed-rank test. There are $2k$ degrees of freedom, where k is the number of P values.

Hybrids were identified in the farm pond populations using RAPD generated species-specific fragments, and the incidence of hybridization detected was compared to that obtained by allozyme analysis. To test for differences in the frequency distribution of fragment presence and absence between species of chambo collected from the wild (i.e. to test the null hypothesis that phenotypes were independently distributed across populations of species), chi-square contingency

values were calculated for each fragment in pair-wise comparisons of *O. karongae*, *O. lidole* and *O. squamipinnis* populations, using the chi-square test of Nass (1959) designed for small expectations and sample sizes. The sum of chi-square tests across all bands was calculated to gain an overall significance of differences in fragment presence and absence between species. The RAPDFST (Black 1995) program was used to compute F_{ST} values between chambo species and between *O. shiranus* and chambo species, based on Weir and Cockerham's Theta (1984) (F_{ST} corrected for small and unequal sample sizes and small number of samples) and Wright's formula (1931) with probability scores to test for significance. Species-specific fragments only were used in this analysis since the program was unable to examine the large number of fragments generated in this study. Separate estimates were also made from the analyses of all fragments scored for each of the four primers individually.

The RAPDPLOT computer package (Black 1995) was used to create pair-wise distance matrices between every individual based on the measure $1\text{-}S_{xy}$ (see formula 4.1). The PHYLIP (Felsenstein 1993) computer package was used to create dendrograms from the distance matrix (of all fragments analysed) of the four species collected from the wild (*O. shiranus*, *O. karongae*, *O. squamipinnis* and *O. lidole*) including seven individuals of unknown identity, and the two reference species (*O. aureus* and *O. mossambicus*) using the nearest neighbour-joining procedure (Saitou & Nei 1987). This was compared to a dendrogram of the same individuals produced using an unweighted pair group method algorithm (UPGMA) (Sneath & Sokal 1973). To establish the ability of different fragments to separate the species analysed, neighbour-joining trees were generated using distance matrices based only on species-specific diagnostic fragments, and based only on non-diagnostic fragments.

In order to visualise the relationship between individual wild fish and individual farm pond fish ('pure' and hybrid individuals), neighbour-joining trees were constructed for each farm sampled (Domasi Aquaculture centre, Dwangwa sugar corporation and Mzuzu Aquaculture centre) with the four species of wild fish. The relationship between all the farm ponds was also analysed by constructing a neighbour-joining tree from the distance matrix. In order to construct a tree of all 13 populations sampled (four wild, seven farm pond and two reference species) the mean genetic divergence (Nei & Tajima 1981) between all pairs of populations was calculated from the distance matrix of all individuals using the DA program in the REAP (McElroy *et al.* 1991) computer package. The DA program could analyse only 100 individuals at a time, so the distance matrix of 192 individuals was randomly sampled three times and a mean genetic divergence for

each population calculated.

The correspondence between distance matrices produced by each RAPD primer was tested using the MXCOMP program of the NTSYS (Rohlf 1992) computer package. The test criterion is

$$Z = \sum_{i,j} X_{ij} Y_{ij}, \qquad \textbf{(4.3)}$$

where X_{ij} and Y_{ij} are the off-diagonal elements of matrices X and Y (Mantel 1967). If the two matrices contain corresponding similarity estimates, then Z will be larger than that expected by chance. The estimated Z was compared with its permutational distribution obtained from 100 random samples of all possible permutations of the matrices. This provided an empirical probability of obtaining a random Z in excess of the estimated Z. A Pearson product-moment correlation (r) (or Normalized Mantel Statistic) between the elements in the two matrices was also computed (Smouse *et al.* 1986). The correspondence between allozyme and RAPD-based genetic distance matrices was tested for using the MXCOMP program. The distance matrices were based on 100 individuals that had been used in both types of analyses. Two genetic divergence matrices (Nei & Tajima 1981) were estimated from distance matrices based on only diagnostic RAPD fragments and on all RAPD fragments analysed, using the DA program in the REAP computer package (McElroy *et al.* 1991). These were both tested against a genetic distance (Nei 1978) matrix estimated from the analysis of 13 enzyme loci (Chapter 3).

4.3 RESULTS

4.3.1 Preliminary investigations - *The power and reliability of the RAPD technique*

4.3.1.1 Detecting variation in the RAPD banding patterns of wild and inbred lines of O. spilurus

The preliminary study of inbred and wild lines of tilapia (*O. spilurus*) using RAPD analysis support the intuitive result that inbred lines show a higher degree of similarity than wild lines (Table 4.5). Mean S.I. values, produced from the analyses of nine RAPD primers, were progressively higher as the level of inbreeding increased (i.e. lowest mean S.I. was obtained for the wild line, and the highest mean S.I. was obtained for the F_3 line), although values obtained for individual primers did not always show this pattern (Table 4.5). The S.I. values obtained for the wild line were significantly lower than from those obtained for the F_3 line (Table 4.6). In a combined probabilities test, the wild line was significantly different from all other lines (X^2 = 14.832, with 6 d.f., P<0.025). The S.I. values obtained for the 'unknown' Stirling line were not found to differ significantly from those of the wild, F_2 or F_3 lines (Table 4.6). However, the least

significant difference was found between the Stirling and F_2 line, suggesting that the Stirling fish are not directly from the wild and may be of second generation.

Table 4.5 Mean Similarity Index (S.I.) for each RAPD primer and across all nine primers for the four lines of *O. spilurus* analysed (Wild, F_2 generation, F_3 generation and unknown Stirling line), and the number of bands scored at each primer (188 bands scored in total).

Primer	no. of bands scored	Mean S.I. for each *O. spilurus* line			
		F_2 n=6	Wild n=2	Stirling n=2	F_3 n=6
OPA 08	20	0.895	0.903	0.812	0.923
OPA 11	11	0.971	0.941	1.000	1.000
OPB 06	33	0.985	0.909	0.958	0.989
OPB 07	20	1.000	0.966	1.000	0.983
OPB 12	33	0.964	0.941	0.917	0.979
OPC 01	20	1.000	1.000	1.000	1.000
OPC 02	14	0.968	1.000	1.000	0.976
OPC 05	21	0.980	0.909	1.000	0.986
OPC 10	16	0.948	0.909	0.956	0.958
Overall mean S.I.		0.968	0.942	0.960	0.977

Table 4.6 The number of cases, out of nine comparisons, where the mean Similarity Index (S.I.) value at each primer for one line of fish is greater than (>), less than (<) or equal to (=) the other line of fish in the comparison. And the results of a Wilcoxon match-pairs signed-rank (*z* value and probability) for each comparison.

Test †	>	<	=	*z*	*P*
F_2 'vs' Wild	6	2	1	-1.820	0.069
F_3 'vs' Wild	7	1	1	-2.100	0.036
F_2 'vs' F_3	1	7	1	-1.680	0.093
F_2 'vs' Stirling	3	4	2	-0.338	0.735
F_3 'vs' Stirling	4	3	2	-0.845	0.398
Wild 'vs' Stirling	2	5	2	-1.183	0.237

† Lines of *O. spilurus* compared: Wild population; F_2 generation; F_3 generation; Unknown Stirling line.

4.3.1.2 Reproducibility of amplification products

No visual differences were apparent in the banding patterns of individuals where the genomic DNA was amplified more than once (in different reaction mixtures) and compared side-by-side on the same gel. PCR products of the same individual that were run on different gels varied in banding pattern only when there were differences in running time, although additional bands were not observed. The intensity of the same band varied between gels if silver-staining times varied slightly, although the relative intensity between bands on the same gel remained the same. Banding patterns were not consistent when different brands of *Taq* polymerase were used. Fewer bands were observed when *Taq* polymerase supplied by Boehringer-Mannheim was used compared

to when 'Red Hot' polymerase (Applied Biotechnologies) was used. This may have been because reaction conditions were not optimised when using the Boehringer-Mannheim *Taq* polymerase.

Banding patterns also varied when DNA was pooled from several individuals. The population pooling strategy fails to detect low-frequency allelic variation within taxa, since some polymorphisms are not amplified in RAPD reactions due to competition among different genomic DNA templates. Therefore, obtaining a 'cumulative genotype' representative of the population being studied requires equal proportions of the individual DNA templates. In this study, inconsistency in the banding patterns of pooled samples may have been due to varying proportions of individual DNA templates in the pooled sample caused by the inaccurate quantification of DNA. That is, either the quantification of DNA was not accurate, or the template DNA was not mixed evenly within the buffer solution when it was being quantified or evenly within the pooled DNA. Therefore, the DNA of each fish was always analysed individually.

4.3.1.3 Repeatability of band scoring

The consistency of scoring was tested by analysing the same gel three times, produced using primer OPA 08 during the preliminary analyses. In pair-wise comparisons between all three analyses, a maximum of 1.4% of fragments scored across the gel (of 14 lanes) were scored differently (as present rather than absent, and vice-versa) (Table 4.7). The majority of lanes had no mismatches, and a maximum of 5% (1 out of 20) mismatches per lane were found. All discrepancies in scoring occurred in the upper half of the gel (over 1230 bp), where fragments tend to be closely packed. Therefore, to ensure good band separation, gels were run for a relatively long time (up to 4.5 hours) during electrophoresis. To ensure the precise analyses of fragments, only those below 1750 bp were scored.

Table 4.7 Repeatability in band scoring of RAPD gels. 280 scores were made per gel based on the presence or absence of bands (20 scores per lane, across 14 lanes). Gels were analysed three times; discrepancies are presented as the maximum percentage of mismatches between scores for a single lane and the percentage of mismatches across 14 lanes.

Band scoring analyses compared	Discrepancies in scoring between analyses*		Maximum % mismatches for a single lane	% mismatches across all lanes
	P/A	A/P		
1st 'vs' 2nd	2	2	5.0%	1.4%
2nd 'vs' 3rd	2	0	5.0%	0.7%
1st 'vs' 3rd	2	0	5.0%	0.7%

* P/A, number of bands which were scored as present in the 1st analyses but as absent in the 2nd analyses; A/P, number of bands which were scored as absent in the 1st but as present in the 2nd analyses. (Comparisons also between 2nd and 3rd analyses, and between 1st and 3rd analyses).

4.3.2 Quality of fin tissue samples collected in Malawi

Many fin tissue samples collected for RAPD analysis were not usable because the extracted DNA was degraded. The number of useable fin tissue samples (66.6%) was far lower than the number of useable muscle and liver tissue samples (96%) collected for allozyme analysis (See Table 3.2, Chapter 3). The greatest losses occurred in samples collected from sites around Lake Malawi (trawl south of Boadzulu Island (21% usable), Maldeco fisheries (53%) and Macawa (14%)) and those collected at Dwangwa (56%). At all of these sites the samples collected had to be transported, for up to an hour in the heat, before they could be processed. Priority was given to preserving tissue for allozyme electrophoretic analyses, which was thought to be most affected by the heat. The length of time before the fin tissue was preserved appears to be the most probably cause of degraded DNA, rather than the method used for preservation (20% DMSO, salt saturated solution) since all samples were useable at sites where they were processed immediately (Domasi and Mzuzu). The losses incurred greatly reduced the sample sizes of the four species from the wild, therefore the fish collected from the garden pond at Chirombo were used to represent two of the wild species (*O. karongae* and *O. shiranus*). There was no obvious morphological evidence, based on colouration and number of anal fin spines, to suggest that these fish were hybrids. Conditions in the pond would not encourage hybridization either because good water clarity was maintained through regular cleaning, the pond was not heavily populated, the ratio of the two species present were approximately equal, and they had not been together in the pond for many breeding seasons. In neighbour-joining cluster analysis the fish collected from the garden pond did not group separately from those collected directly from Lake Malawi or Lake Malombe.

4.3.3 The search for species-specific RAPD markers

RAPD analyses with four random decamer primers revealed four marker fragments that were specific to *O. shiranus*, of which three were always present (OPA 08 $_{460}$, OPA 08 $_{370}$ and OPE 15 $_{890}$), and one (OPG 17 $_{462}$) that was not detected in all *O. shiranus* fish, but was never found in the chambo species (i.e. amplification site and polymorphism unique to *O. shiranus*). Five marker fragments were specific to all chambo species, of which two were always present (OPA 10 $_{320}$ and OPG 17 $_{635}$) and three (OPA 08 $_{515}$, OPE 15 $_{784}$ and OPG 17 $_{478}$) that were not observed in all chambo fish, but were unique to these species (Figure 4.1 to 4.4). No fragments were found that were specific to a single chambo species, which meant that the individuals of unknown identity could not be identified. A large number of fragments were scored in an attempt to detect

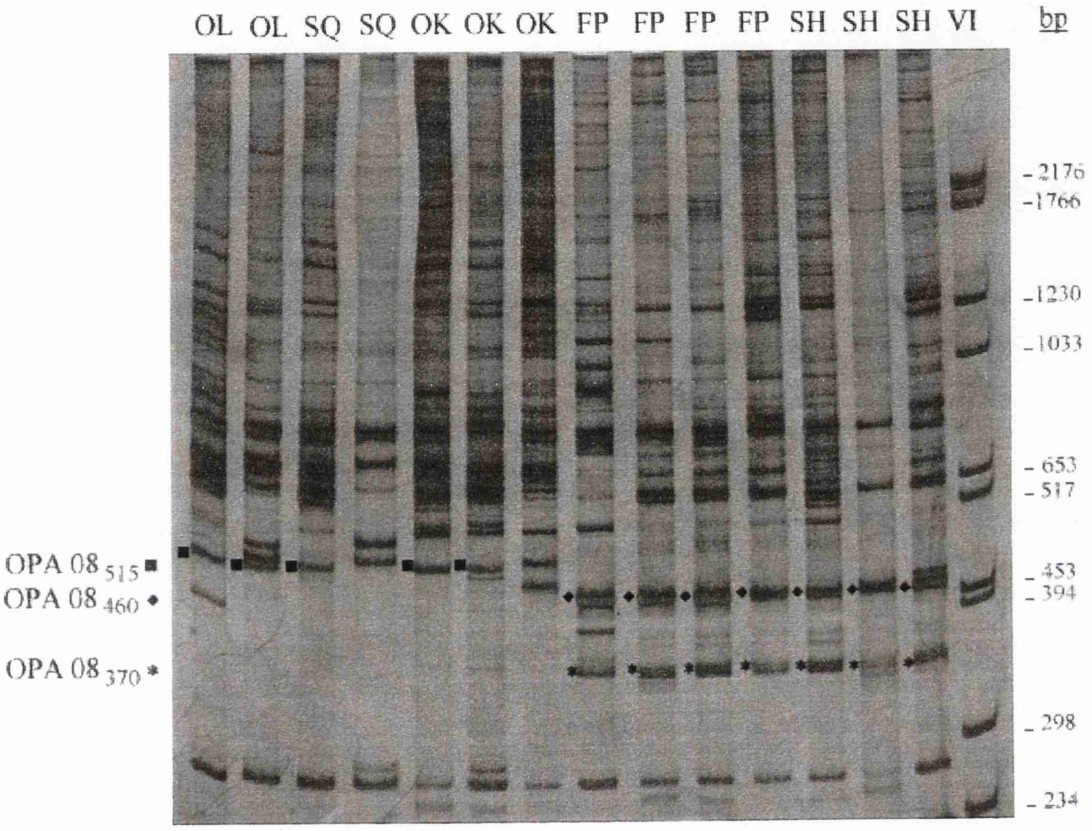


Figure 4.1 RAPD amplification with primer OPA 08
■ Marker OPA 08 $_{515}$ specific to the chambo species (not always present)
Chambo species: OL, *O. lidole;* SQ, *O. squamipinnis;* and OK, *O. karongae*
◆ Marker OPA 08 $_{460}$ specific to *O. shiranus* (always present)
✸ Marker OPA 08 $_{370}$ specific to *O. shiranus* (always present)
SH, *O. shiranus*; FP, putative hybrid fish from farm pond
VI, Boehringer marker VI; bp, basepair lengths illustrated to right of gel

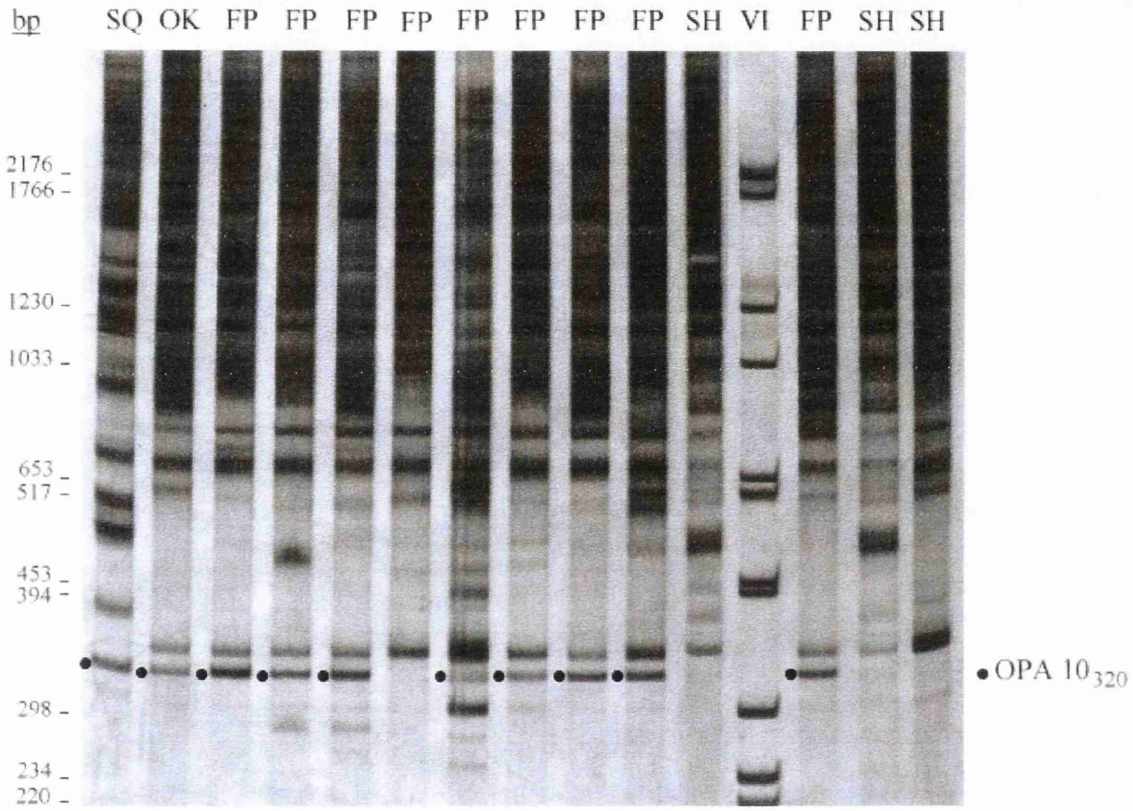


Figure 4.2 RAPD amplification with primer OPA 10

• Marker OPA 10_{320} specific to the chambo species (always present)

Chambo species: SQ, *O. squamipinnis*; OK, *O. karongae*
(*O. lidole* not present on this particular gel)

SH, *O. shiranus*; FP, putative hybrid fish from farm pond

VI, Boehringer marker VI; bp, basepair lengths illustrated to left of gel

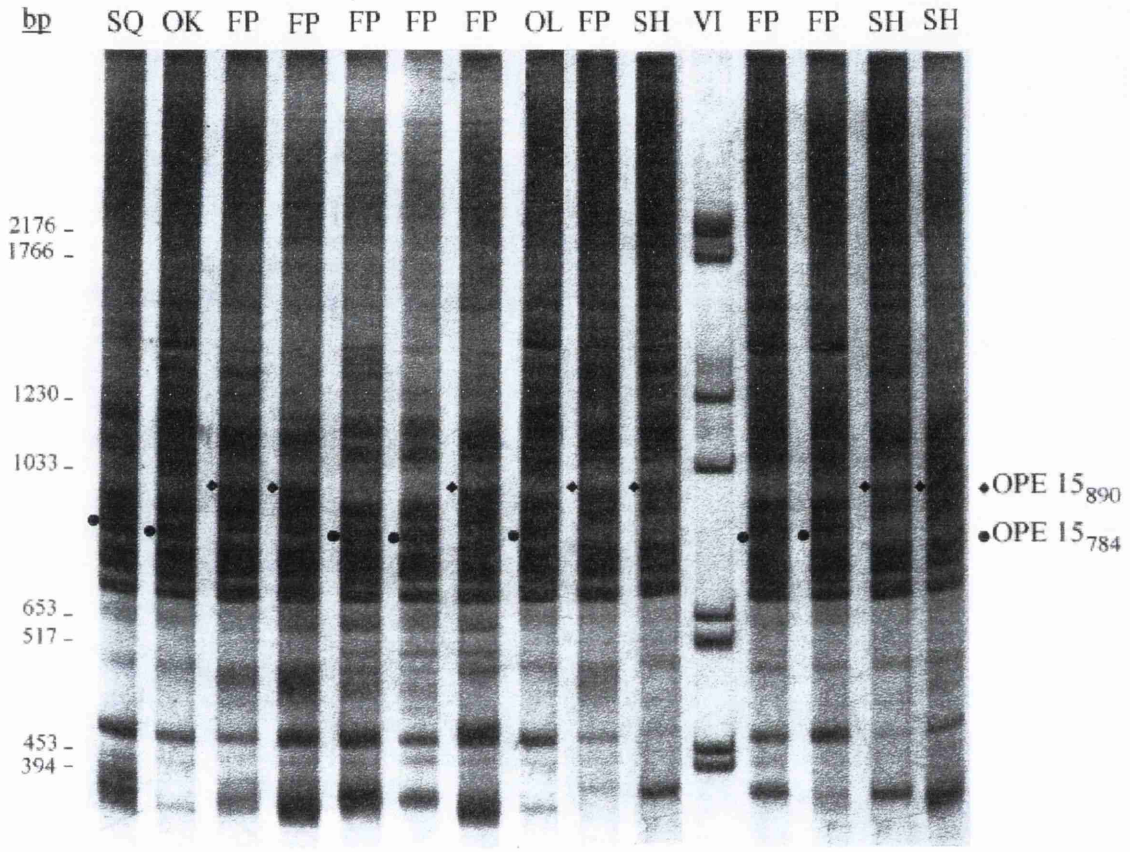


Figure 4.3 RAPD amplification with primer OPE 15

◆ Marker OPE 15 $_{890}$ specific to *O. shiranus* (always present)

SH, *O. shiranus*; FP, putative hybrid fish from farm pond

● Marker OPE 15 $_{784}$ specific to the chambo species (not always present)

Chambo species: OL, *O. lidole;* SQ, *O. squamipinnis;* and OK, *O. karongae*

VI, Boehringer marker VI; bp, basepair lengths illustrated to left of gel

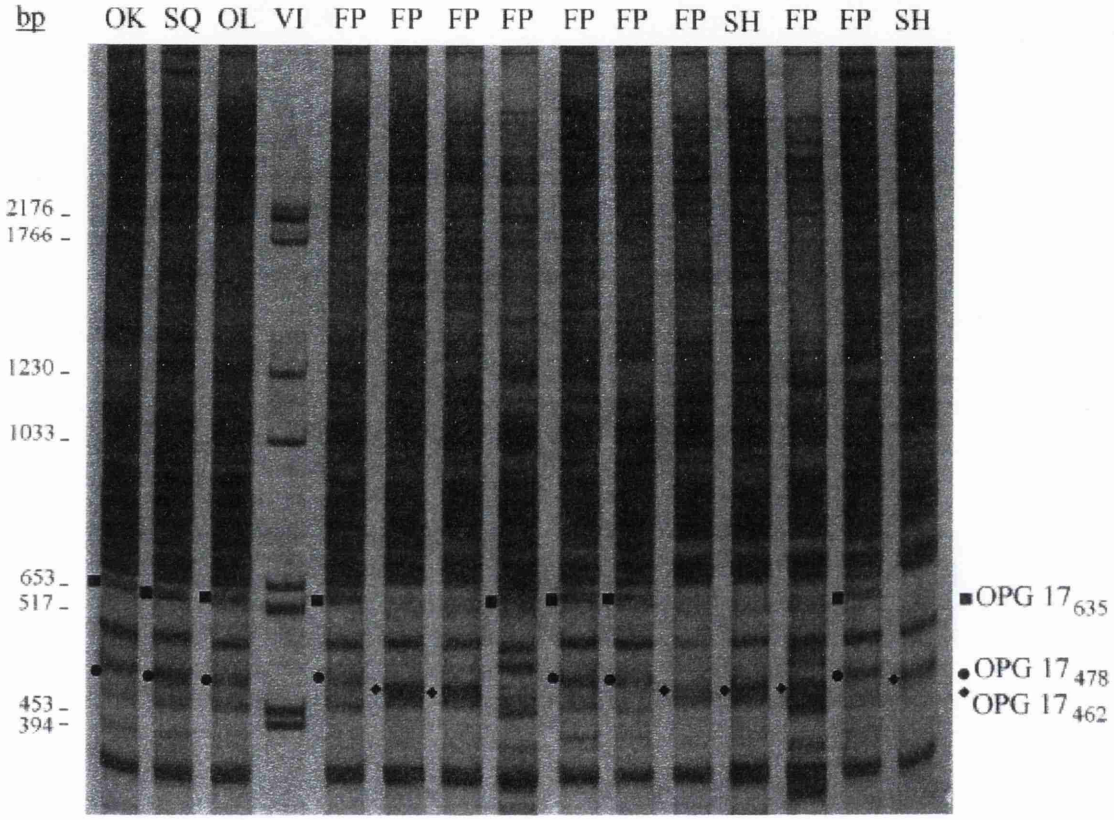


Figure 4.4 RAPD amplification with primer OPG 17

■ Marker OPG 17 $_{635}$ specific to the chambo species (always present)

● Marker OPG 17 $_{478}$ specific to the chambo species (not always present)

Chambo species: OL, *O. lidole;* SQ, *O. squamipinnis;* and OK, *O. karongae*

◆ Marker OPG 17 $_{462}$ specific to *O. shiranus* (not always present)

SH, *O. shiranus*; FP, putative hybrid fish from farm pond

VI, Boehringer marker VI; bp, basepair lengths illustrated to left of gel

differences between the three species of chambo. A total of 163 fragments were scored, using four primers: 43 fragments for primer OPA 08, 57 fragments for primer OPA 10, 21 fragments for primer OPE 15, and 42 fragments for primer OPG 17. In this analysis, three more fragments were found to be unique to *O. shiranus* and one more fragment was unique to chambo species, but all at low frequencies (Table 4.8). Seven fragments where found in relatively high frequencies in the farm ponds when compared to their frequencies in the wild populations. The fragment OPA 10_{396} was unique to the farm pond populations (Table 4.8).

Table 4.8 RAPD fragments which were either unique to certain groups, or found in relatively high frequencies in some farm pond samples. Expressed a percentage of fish in each group which had a particular fragment.

Fragment	OSHI n = 20	CH n = 40	DM n = 30	DW n= 45	MZ n = 43
OPA 10_{582}†	5.0	0.0	20.0	17.8	16.3
OPA 10_{465}	5.0	2.5	6.7	13.3	2.3
OPA 10_{405}	5.0	5.0	10.0	13.3	0.0
OPA 10_{396}*	0.0	0.0	6.7	11.1	4.6
OPE 15_{635}†	15.0	0.0	23.3	11.1	2.3
OPG 17_{547}♯	0.0	7.5	3.3	11.1	0.0
OPG 17_{522}†	10.0	0.0	0.0	17.8	0.0

† Fragment unique to *O. shiranus;* ♯ Fragment unique to chambo species; * Fragment unique to farm populations; OSHI, *O. shiranus*; CH, chambo; DM, Domasi farm ponds; DW, Dwangwa farm ponds; MZ, Mzuzu farm ponds.

4.3.4 SCAR and SSCP analysis

No fragments were amplified from DNA samples, including the *O. aureus* control, using the SCAR primer OPA 13_{310}. Amplification products of primer OPA 13_{590} were produced only in samples when the reaction mixture contained 2 μl $MgCl_2$ and when the annealing temperature was lowered to 54°C. Variation was detected only in *O. aureus*, which gave a smaller fragment than *O. shiranus* and chambo. The non-stringent reaction conditions resulted in many non-specific fragments, and so the amplification products were not used in SSCP analysis. No variation was found when amplification products of SCAR primer OPB 06_{374} were run on a 1.5% agarose gel, although variation in the fragment size of *O. aureus* was detected on a 6% polyacrylamide (Figure III.1, Appendix III). SSCP analysis of the amplification products from primer OPB 06_{374}, run on a 10% polyacrylamide gel for 5 hours, revealed very little variation. All individuals were homozygous, except for two fish (an *O. lidole* fish and a DWST pond fish) which were both heterozygous for the same alleles. The *O. aureus* dsDNA fragment was smaller, but there appeared to be no difference in the ssDNA fragments when compared to the other fish, possibly because of the contraction of scale at the top of the gel (Figure III.2, Appendix III).

4.3.5 Incidence of hybridization

If an individual fish was found to have at least one *O. shiranus* diagnostic fragment (out of a possible four) and one chambo diagnostic fragment (out of a possible five), then it was assigned a hybrid status. Hybrids between chambo and *O. shiranus* were found in all seven farm ponds sampled. The highest frequency of hybrids was observed in samples from the *O. shiranus* pond at Domasi and the two ponds at Dwangwa (Table 4.9). These results mirror those found using allozymes (Chapter 3), although the percentage of hybrids detected using RAPD was lower (Table 4.10). This lower percentage may, however, be due to the different numbers of fish analysed by the two methods, rather than a reflection on the ability of RAPD to detect hybrids. Of the ponds investigated using allozymes (all ponds in Table 4.9, except MZKB and MZSH), none contained more than one type of 'pure' species, whereas RAPD analysis revealed the presence of both *O. shiranus* and chambo in the DWST pond. Both species, and hybrids, were also found in the *O. karongae* breeding pond and *O. shiranus* holding pond at Mzuzu. As with allozymes, the frequency of hybrids detected shown in Table 4.9 are the minimum frequency of hybrids, since individuals with 'genotypes' of pure chambo or *O. shiranus*, may in fact be later generation hybrids. A number of the fish designated as *O. shiranus* (six fish) or chambo (eight fish) did not possess all of the species diagnostic fragments that were fixed in all *O. shiranus* (three fragments) or all chambo (two fragments) fish. These individuals may be later generation backcross hybrids, in which case the number of hybrids in five of the farm ponds would increase (see revised estimates in Table 4.9).

Table 4.9 Frequency of chambo, *O. shiranus* and hybrid fish in farm ponds, based on nine RAPD marker fragments generated using four primers. Revised estimates in parenthesis, based on the absence of some specific diagnostic fragments (see text for details).

Farm* pond	no.	Chambo	*O. shiranus*	Hybrids
DMSH	15	0.0	33.3 (13.3)	66.7 (86.7)
DMOK	15	93.3 (80.0)	0.0	6.7 (20.0)
DWSE	27	0.0	44.4	55.6
DWST	18	16.7 (11.1)	27.8 (22.2)	55.6 (66.7)
MZKH	10	80.0	0.0	20.0
MZKB	18	88.9 (72.2)	5.6 (0.0)	5.6 (27.8)
MZSH	15	40.0 (26.7)	53.3 (46.6)	6.7 (26.7)

* DMSH, *O. shiranus* pond Domasi; DMOK, *O. karongae* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storage tank pond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu; MZKB, *O. karongae* breeding pond Mzuzu; MZSH, *O. shiranus* holding pond Mzuzu.

It was not possible to determine if any hybrids were F_1 hybrids, although no individuals were found to possess all the diagnostic fragments of chambo and *O. shiranus*. The combination of *O. shiranus* and chambo diagnostic fragments in hybrid individuals of each farm pond, largely reflect the species (*O. shiranus* or chambo) which were chosen for stocking and therefore the direction of hybrid backcross. For example, hybrid individuals from the *O. shiranus* farm pond at Domasi (DMSH) all possessed more *O. shiranus* than chambo fragments, whereas both hybrid individuals from the *O. karongae* holding pond at Mzuzu (MZKH) had more chambo fragments. 50% of hybrid fish from the storage pond at Dwangwa (DWST) had an equal number of *O. shiranus* and chambo fragments (Table III.1, Appendix III).

Over 40% of the fish analysed by both RAPD and allozymes, were designated a different identity by the two methods (Table 4.10). More individuals were identified as hybrids using allozymes, although the results between the two methods may agree more than at first expected. Three of the fish identified as *O. shiranus* with the diagnostic loci of allozyme analysis, and as hybrids with RAPD, did in fact possess alleles unique to a chambo species (*EST*93* or *EST*77*). Furthermore, four of the fish identified as *O. shiranus* with RAPD, and as hybrids with allozymes, did not have all of the fixed *O. shiranus* fragments. If it is assumed that individuals identified as hybrids, by either method, are identified correctly, then the frequency of hybrids found in four of the farm ponds greatly increases than when analysed with one method alone. Based on this assumption, over 93% of fish in the storage pond at Dwangwa (DWST) were hybrids (Table 4.10).

Table 4.10 The incidence of hybridization in Malawi farm ponds based RAPD and allozyme analysis, including: the number of discrepancies in identification based on the two methods; the total number of fish analysed using either method; and the maximum percentage of hybrids found in each pond based on the combined results of RAPD and allozyme analysis.

Farm pond	Discrepancies in the identification of fish analysed with both RAPD (1) and allozymes (2)*					Incidence of hybridization based on both analysis	
	No. of fish	1)Chambo 2)Hybrid	1)*O. shiranus* 2)Hybrid	1)Hybrid 2)Chambo	1)Hybrid 2)*O. shiranus*	Total no. of fish analysed	Percentage of hybrids
DMSH	10	0	3	0	2	15	78.6
DMOK	9	1	0	0	0	15	13.3
DWSE	27	0	8	0	4	30	83.3
DWST	18	1	5	0	1	48	93.8
MZKH	10	3	0	2	0	10	50.0

* Number of fish analysed with both allozymes and RAPD, and the number of discrepancies in their identification (Chambo, hybrid or *O. shiranus*) using the two methods, where: 1), identification based on RAPD analysis; 2) identification based on allozyme analysis. For results on the percentage of hybrids estimated from RAPD and allozyme analysis alone, see Table 4.9 and Table 3.6 (Chapter 3), respectively.

4.3.6 Genetic differentiation between chambo species, and *O. shiranus*

The distribution in the frequencies of fragment presence and absence, across all fragments analysed, in *O. karongae* was significantly different from that in *O. squamipinnis* ($P<0.001$) and in *O. lidole* ($P<0.005$), although there was no significant difference between *O. lidole* and *O. squamipinnis* (Table 4.11). However, some significant differences were observed at individual fragments in all pair-wise comparisons (Table III.2, Appendix III). Of the 163 fragments scored, 19.6% were significantly different in their frequencies of presence and absence, over all three pair-wise comparisons between species. The primer OPA 08 was most effective in detecting genetic differentiation because over 25% of fragments scored revealed a significant difference. Primer OPE 15 revealed the least differences (14.3%). These analyses were not conducted between the chambo and *O. shiranus*, because they could be clearly separated using the nine RAPD markers.

Table 4.11 Pair-wise tests of the distribution of RAPD 'genotypes' across chambo species. Results of Chi-square (Nass 1959) analyses of individual fragments: number of fragments analysed, number of fragments where the difference in frequencies are significant ($P<0.05$) and the percentage of all fragments scored (out of 163) where significant differences were found between species. Total Chi-square value of all RAPD fragments analysed, total degrees of freedom and the significance of difference in the distribution of fragments between species.

Pair-wise comparison	Analyses of individual RAPD fragments			Analyses across all fragments		
	no. fragments analysed *	no. fragments where $P<0.05$	% fragments where $P<0.05$	Chi-square	degrees of freedom	P value
OKAR / OSQU	149	24	14.7	423.776	223.417	<0.001
OKAR / OLID	139	9	5.5	192.233	143.683	0.004
OSQU / OLID	132	5	3.1	145.424	150.291	0.450

* Number of fragments analysed: i.e. those where a difference in the frequency of presence and absence occurred between species (details of Chi-square test results for fragments individually in Table III.2, Appendix III).
OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*.

The level of genetic differentiation observed between the three chambo species (F_{ST} = 0.049, Weir & Cockerham's Theta method (1984), estimated using diagnostic RAPD fragments only), was far lower than that observed between *O. shiranus* and chambo (F_{ST} = 0.912) (Table 4.12). The values were similar to those obtained by allozymes (F_{ST} = 0.067 and 0.527, respectively), although the value for *O. shiranus* and chambo was greater. F_{ST} values estimated from the data of each primer alone, revealed contrasting results. In three of the four primers (OPA 08, OPA 10 and OPG 17), lower F_{ST} values were observed between the chambo species than between chambo and *O. shiranus*. Values given for chambo species, when the data of each

Table 4.12 Estimates of F_{ST} values for *O. shiranus* and chambo species from the analyses of nine species diagnostic fragments alone (from four RAPD primers), and from the analyses of all fragments scored for each of the four primers separately (number of fragments in parenthesis). Estimates based on Weir & Cockerham's Theta method (1984) and Wrights method (1931) with probability values.

	Method	All four primers †	OPA 08 only (43)	OPA 10 only (57)	OPE 15 only (21)	OPG 17 only (42)
Chambo species (n = 40)	Theta	0.049	0.351	0.016	0.583	0.553
	Wright	0.058	0.132	0.036	0.285	0.295
	P value	0.031	<0.001	0.001	<0.001	<0.001
Chambo and *O. shiranus* (n = 60)	Theta	0.912	0.401	0.052	0.587	0.553
	Wright	0.800	0.181	0.068	0.305	0.293
	P value	<0.001	<0.001	<0.001	<0.001	<0.001

† Three fragments in the analyses of chambo species, and nine fragments in the analyses of *O. shiranus* and chambo.

primer was analysed alone, were generally higher (ranged from 0.016 to 0.583) than those obtained from the analyses of diagnostic fragments only, due to the variability detected by all the other fragments scored. However, F_{ST} values for *O. shiranus* and chambo were all lower (ranged from 0.052 to 0.587) than those obtained from the analyses of diagnostic fragments alone. Primer OPG 17 revealed no difference in estimates of genetic differentiation between tests where chambo species were analysed alone and when all four species were analysed (Table 4.12). Estimates based on Wright's method (1931) revealed similar results for the analyses based on diagnostic fragments only, although the F_{ST} values estimated for each primer (all fragments) were often half as great as those estimated using Weir & Cockerham's Theta method (1984).

Neighbour-joining cluster analysis of chambo and *O. shiranus*, clearly illustrates the separation of an *O. shiranus* clade from the three chambo species (Figure 4.5). Despite the genetic differentiation detected between *O. karongae* and *O. squamipinnis*, and between *O. karongae* and *O. lidole*, there was no indication of separation between these species in the cluster analysis. The chambo species formed a number of mixed clades, which were of no assistance in trying to identify the unknown individuals. It is uncertain as to why one individual of *O. karongae* clustered out from the same node as *O. aureus*. *O. mossambicus* and *O. aureus* were greatly differentiated from the four species from Malawi, and showed far less genetic differentiation within themselves (inbred lines) than the fish collected from the wild. An almost identical grouping pattern was produced using the UPGMA method (Figure 4.6), although not all individuals of *O. shiranus* (including an unknown individual suspected to be *O. shiranus*) clustered out with the main *O. shiranus* clade.

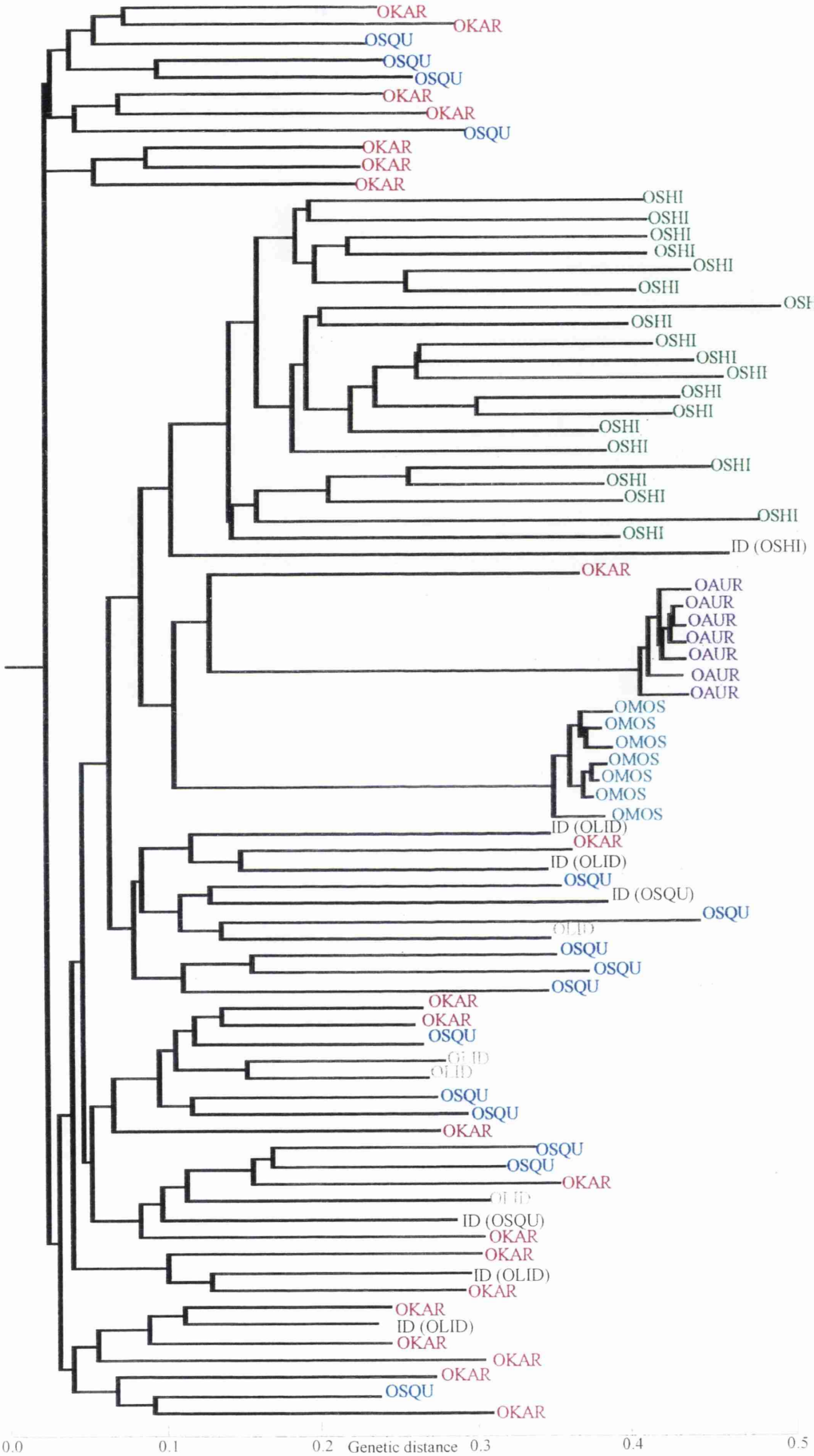


Figure 4.5 RAPD-generated nearest neighbour-joining tree of wild fish from Lake Malawi and Lake Malombe (OSHI, *Oreochromis shiranus;* OSQU, *O. squamipinnis;* OKAR, *O. karongae;* OLID, *O. lidole;* ID, Identification of fish unknown - suspected identity in parentheses) and two reference species (OAUR, *O. aureaus;* OMOS, *O. mossambicus*).

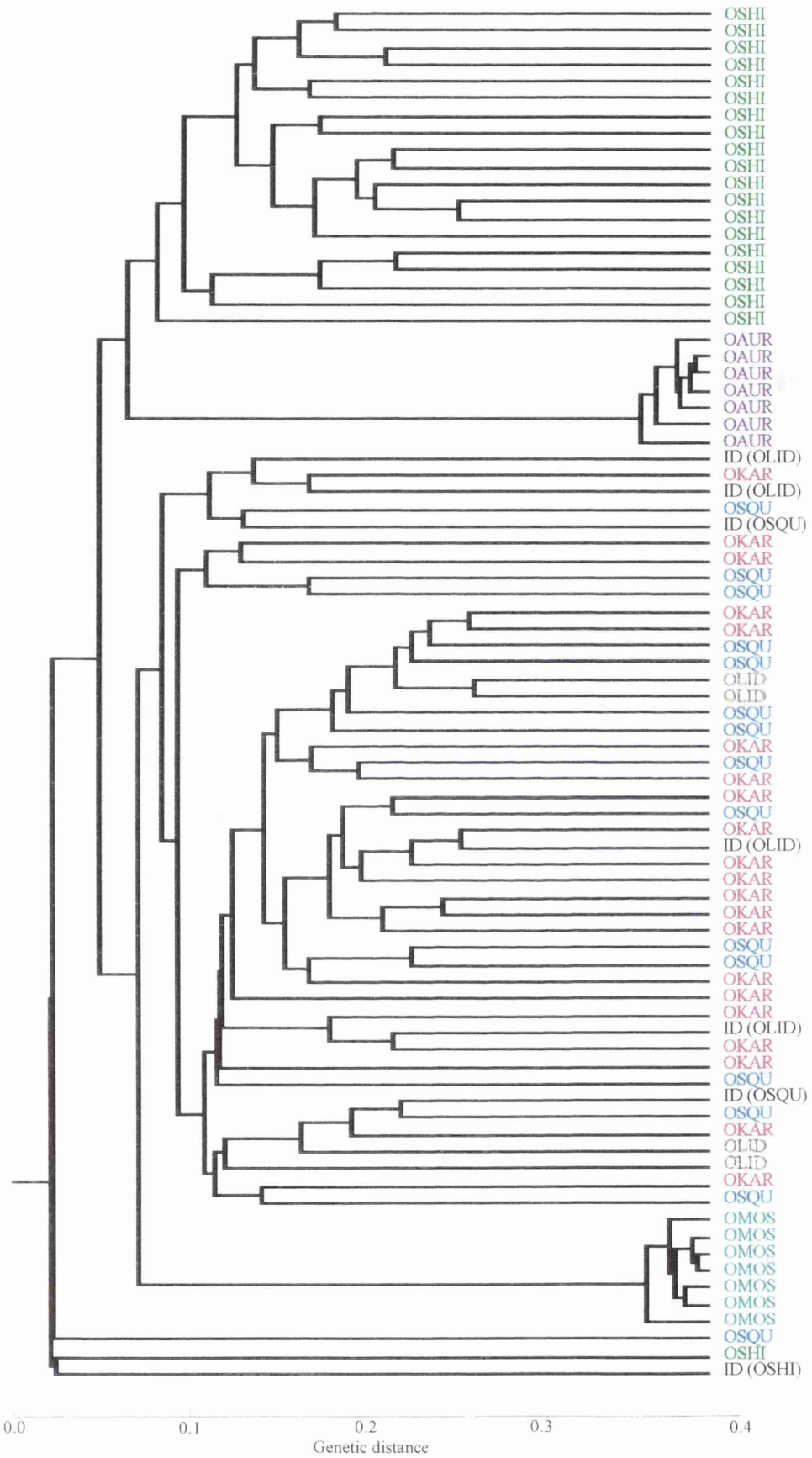


Figure 4.6 RAPD-generated UPGMA tree of wild fish from Lake Malawi and Lake Malombe (OSHI, *Oreochromis shiranus;* OSQU, *O. squamipinnis;* OKAR, *O. karongae;* OLID, *O. lidole;* ID, Identification of fish unknown - suspected identity in parentheses) and two reference species (OAUR, *O. aureaus;* OMOS, *O. mossambicus*).

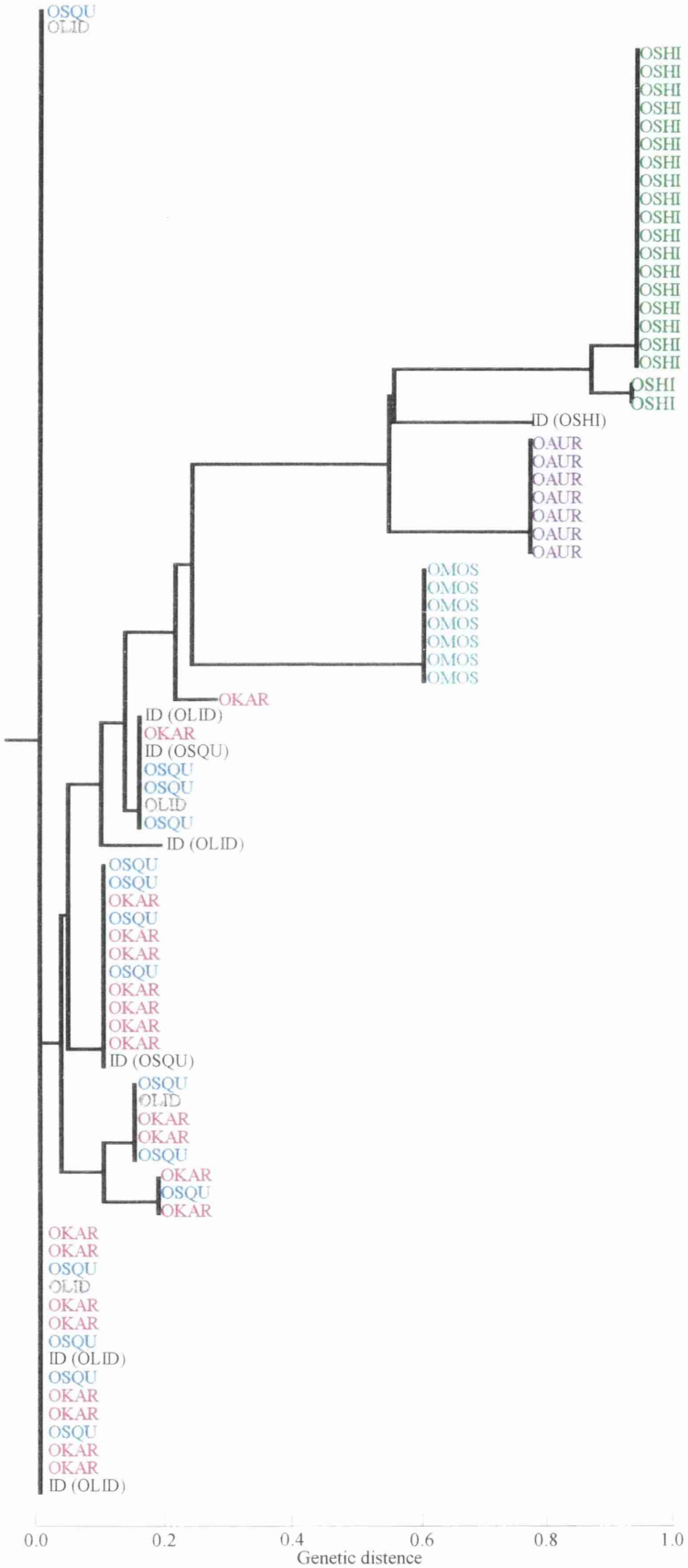


Figure 4.7 RAPD-generated nearest neighbour-joining tree of wild fish (OSHI, *Oreochromis shiranus;* OSQU, *O. squamipinnis;* OKAR, *O. karongae;* OLID, *O. lidole;* ID, Identification of fish unknown - suspected identity in parentheses) and two reference species (OAUR, *O. aureaus;* OMOS, *O. mossambicus*), based on the analyses of nine diagnostic RAPD fragments only.

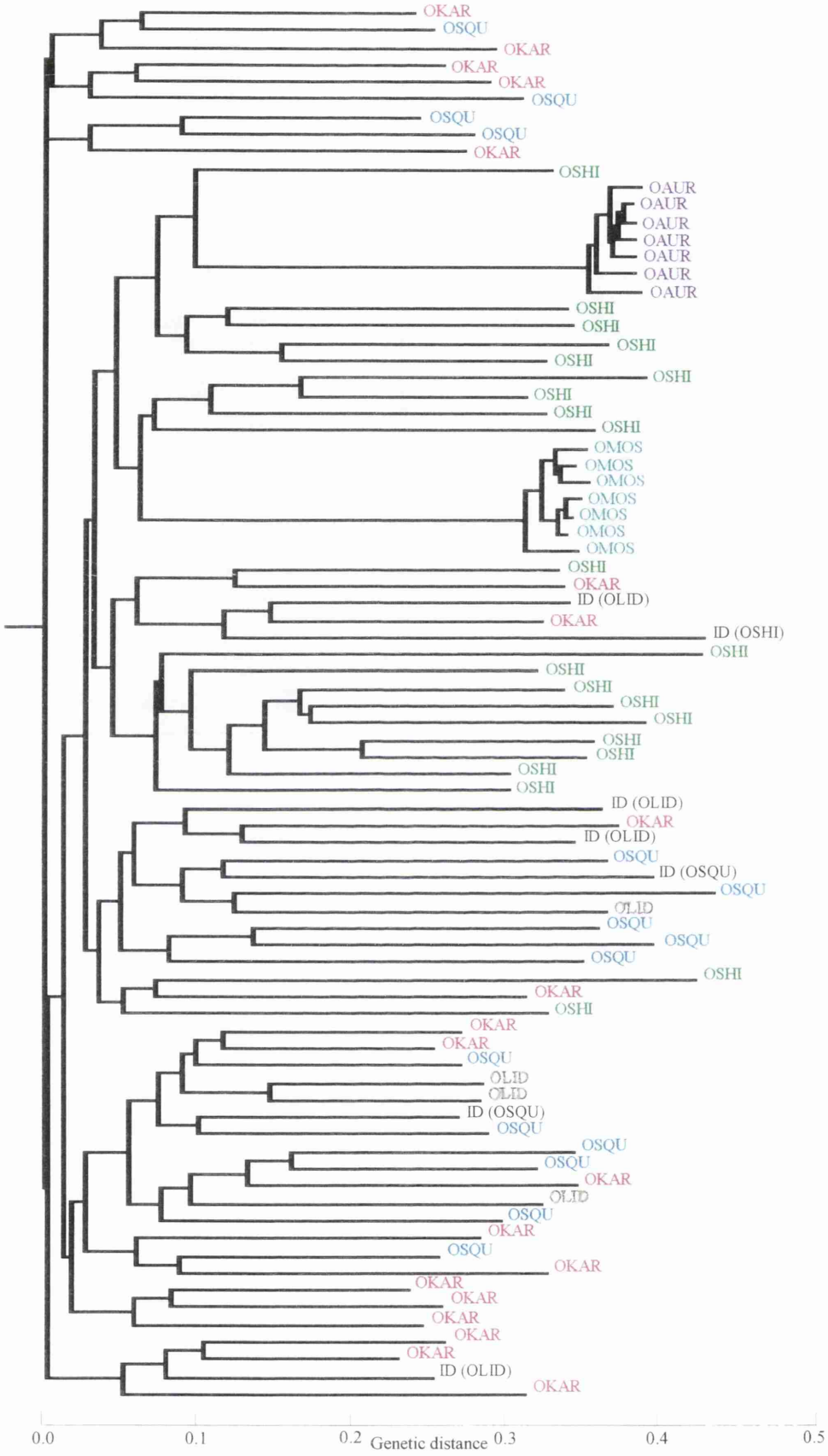


Figure 4.8 RAPD-generated nearest neighbour-joining tree of wild fish (OSHI, *Oreochromis shiranus;* OSQU, *O. squamipinnis;* OKAR, *O. karongae;* OLID, *O. lidole;* ID, Identification of fish unknown - suspected identity in parentheses) and two reference species (OAUR, *O. aureas;* OMOS, *O. mossambicus*), based on the analyses of 154 'non-diagnostic' RAPD fragments.

The separation between the three species of chambo using the UPGMA method was no clearer (Figure 4.6). Since neighbour-joining analyses relies on fewer assumptions then the UPGMA analysis and it generally maximises the differences between species and was generally clearer to visualise, this method was used in all other cluster analyses.

When cluster analysis was performed on the distance matrix of data from nine diagnostic RAPD markers alone (Figure 4.7), the separation of *O. shiranus*, *O. aureus* and *O. mossambicus* was distinct. When compared to the tree generated from all fragments scored, the genetic distance between all species was less, but the species of chambo did not show any structuring. This analysis suggests that separation of the species largely relies on the diagnostic fragments, although when these are removed from the analyses (Figure 4.8) *O. aureus* and *O. mossambicus* cluster out clearly and the *O. shiranus* individuals are still, although to a lesser extent, clustered together.

4.3.7 Genetic relationships between all populations sampled

In the cluster analysis of each farm pond site with wild fish, fish from the ponds in which one species has intentionally been stocked (*O. shiranus* or chambo) tend to cluster within the clade of the relevant species from the wild (Figures 4.9 to 4.11). For example, fish from the *O. shiranus* pond at Domasi (DMSH) cluster exclusively within the same clade as wild *O. shiranus* individuals. Hybrid individuals are also found within this cluster because they are probably progeny from the backcross of hybrids with *O. shiranus* (or of later generation). Fish from the *O. karongae* pond (DMOK), which were largely identified as pure chambo, are scattered throughout most of the clusters of chambo species from the wild (Figure 4.9). Individuals identified as chambo from the three ponds at Mzuzu also cluster with the wild chambo species (Figure 4.11), although they tend to form small clusters of their own, suggesting that these fish may be differentiated from the wild populations (also suggested by the presence of unique fragments, see Table 4.8). The fish from the storage pond at Dwangwa (DWST) also tend to cluster together, and may be regarded as occupying an intermediate position between the wild chambo and *O. shiranus* (Figure 4.10). It is unlikely that these fish are F_1 hybrids, even though some had equal numbers of *O. shiranus* and chambo marker fragments (section 4.3.5), because they were originally taken from the sewage pond as hybrids rather than the intended pure chambo. RAPD analysis did not reveal sufficient variation to separate individuals from different farm pond populations (Figure III. 3, Appendix III), although the fish are clearly grouped according to their identity as *O. shiranus* or chambo, with the hybrid individuals distributed between them.

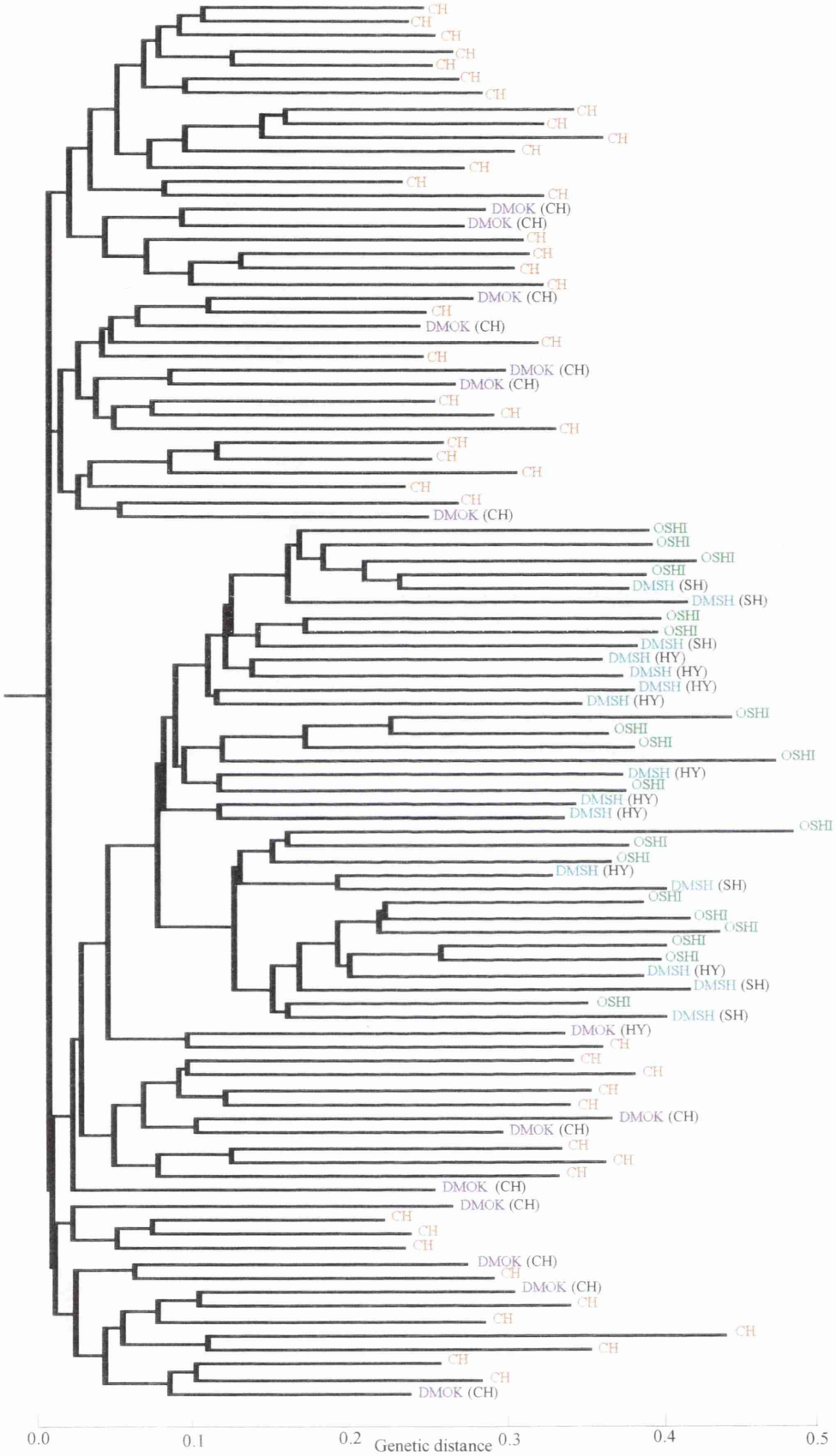


Figure 4.9 RAPD-generated nearest neighbour-joining tree of fish from the *O. karongae* pond (DMOK) and *O. shiranus* pond (DMSH) at Domasi, and wild fish (OSHI, *O. shiranus;* CH, chambo) from Lake Malawi and Lake Malombe. The identity of farm fish, based on nine diagnostic RAPD fragments, is shown in parentheses (SH, *O. shiranus;* CH, chambo; HY, hybrid).

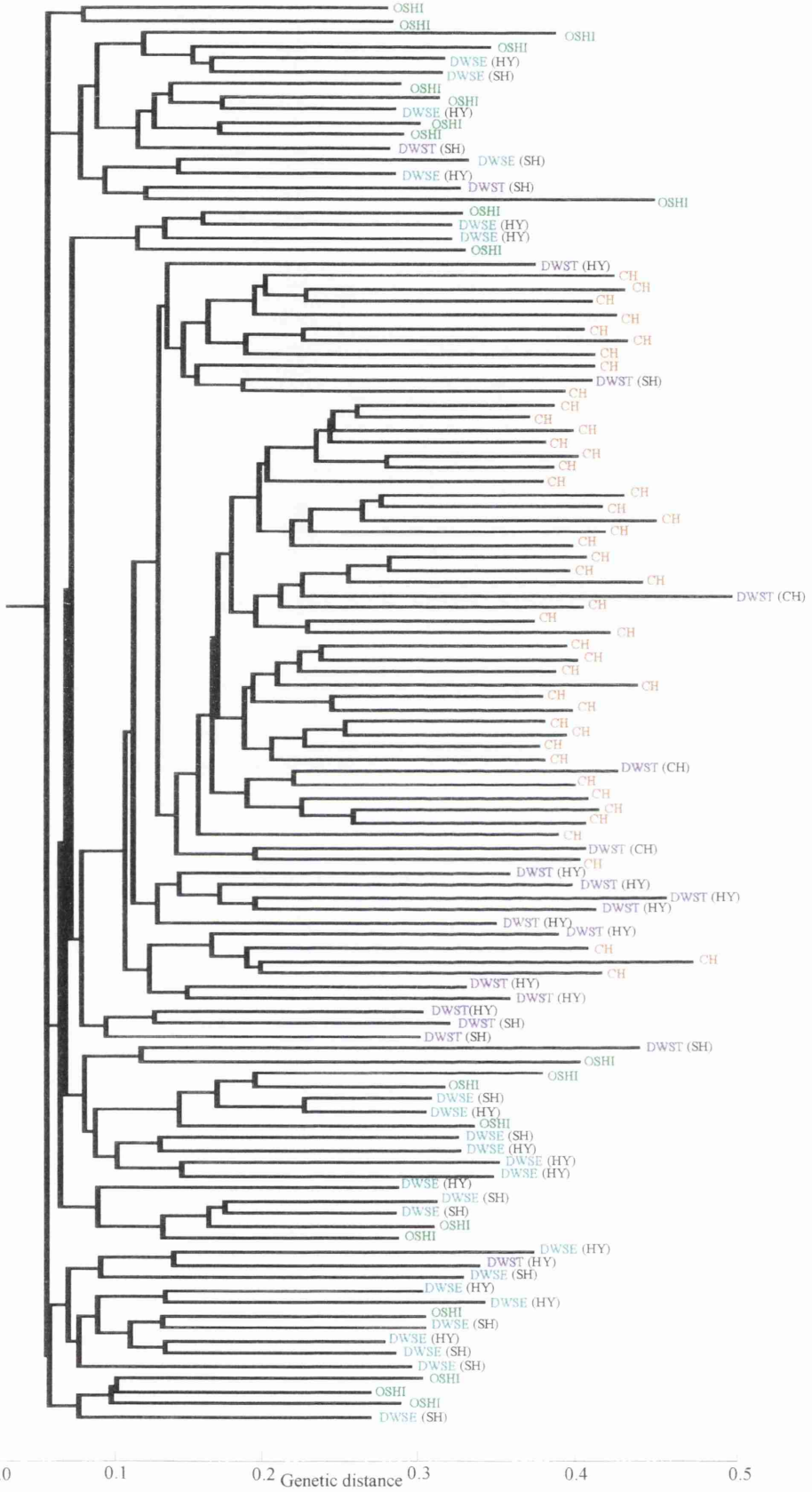


Figure 4.10 RAPD-generated nearest neighbour-joining tree of fish from the Storage tank pond (DWST) and Sewage tank pond (DWSE) at Dwangwa, and wild fish (OSHI, *O. shiranus;* CH, chambo) from Lake Malawi and Lake Malombe. The identity of farm fish, based on nine diagnostic RAPD fragments, is shown in parentheses (SH, *O. shiranus;* CH, chambo; HY, hybrid).

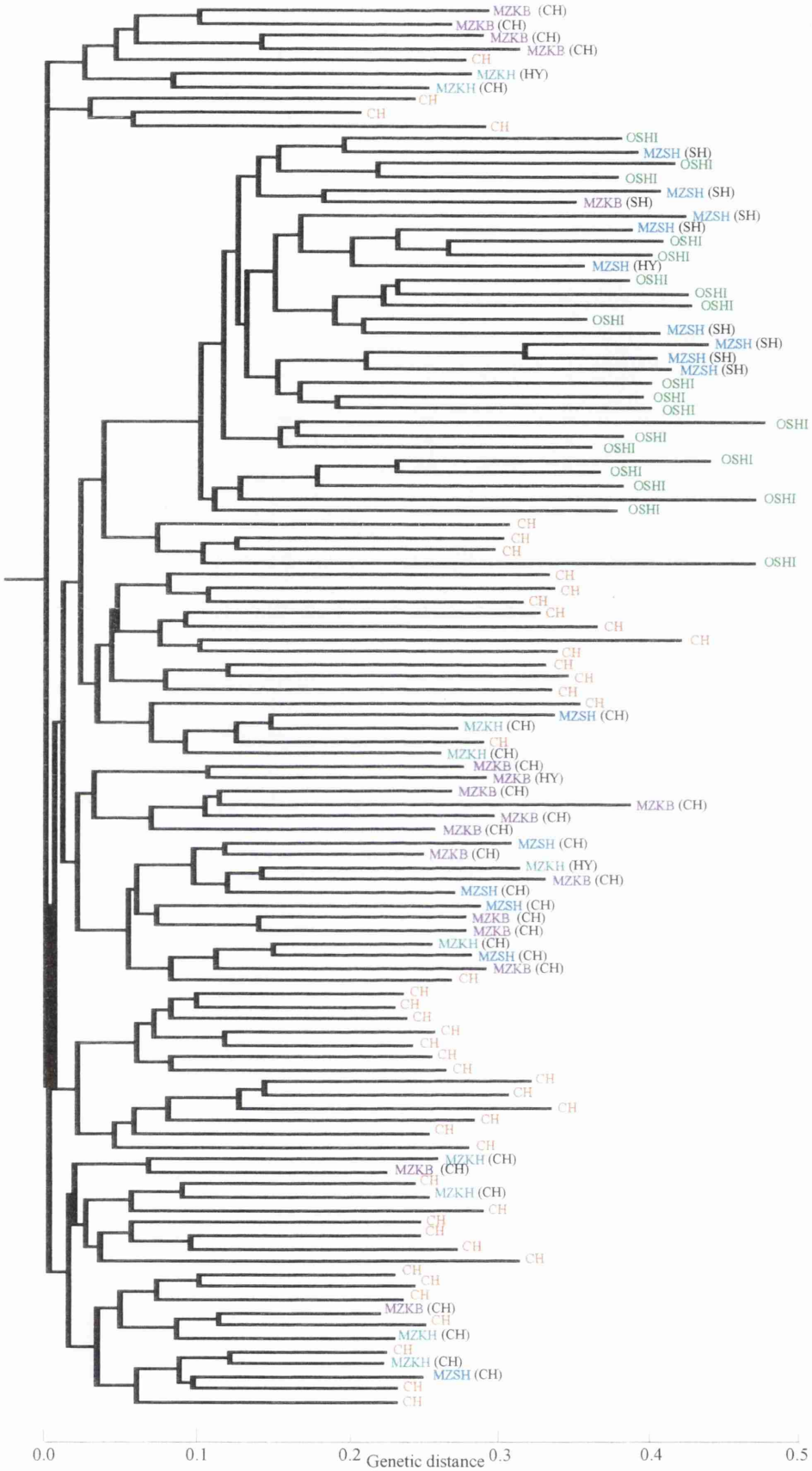


Figure 4.11 RAPD-generated nearest neighbour-joining tree of fish from the *O. karongae* breeding pond (MZKH) and *O. shiranus* holding pond (MZSH) at Mzuzu, and wild fish (OSHI, *O. shiranus;* CH, chambo) from Lake Malawi and Lake Malombe. The identity of farm fish, based on nine diagnostic RAPD fragments, is shown in parentheses (SH, *O. shiranus;* CH, chambo; HY, hybrid).

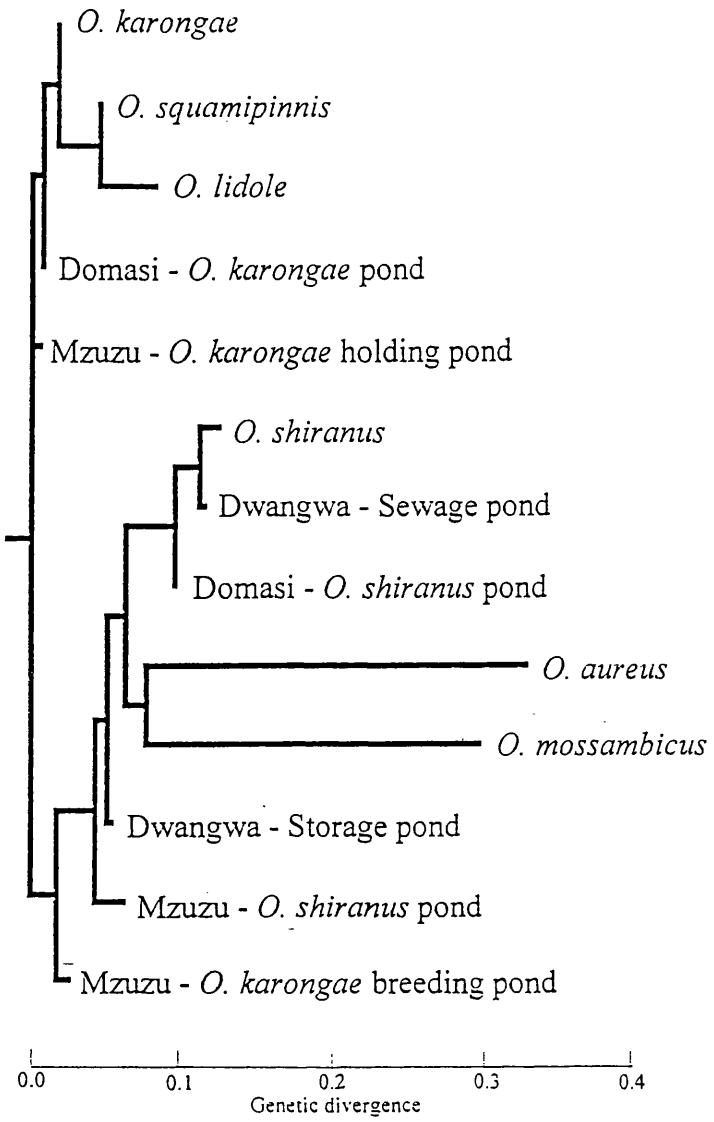


Figure 4.12 Neighbour-joining tree of mean genetic divergence (Nei & Tajima 1981), based on the RAPD analyses of four *Oreochromis* species sampled from Lake Malawi and Lake Malombe, seven farm pond populations sampled in Malawi, and two other species of *Oreochromis* which are not endemic to Malawi.

Overall, the RAPD technique was not sufficiently powerful to discriminate between all individuals of wild and farm pond populations, although cluster analyses of data generated by the technique has been valuable in determining the relationship between wild and farm pond fish. The relationship between all 13 populations analysed, based on the mean genetic divergence (Nei & Tajima 1981) of each population (Table III.3, Appendix III), is represented in Figure 4.12. The position of individual populations obtained from RAPD analysis corresponds well to the positions obtained from allozyme analysis (Figures 4.12 to 4.14). The chambo species clearly form their own clade, where *O. karongae* branches off separately from *O. squamipinnis* and *O. lidole* (reflecting the results in Table 4.11). Neighbour-joining analyses of distances obtained by allozyme analysis,

does not cluster *O. squamipinnis* with *O. lidole*, although the pattern of clustering varied with the measure used to estimate genetic distance. The ponds where a chambo species was chosen for stocking (DMOK, MZOK and MZKB) are positioned closer to the chambo species than to *O. shiranus*. Whereas, the DMSH pond and DWSE clustered with *O. shiranus*. The MZST and MZSH pond, where both 'pure' *O. shiranus* and chambo were observed (Table 4.9), occupy an intermediate position. *O. aureus* and *O. mossambicus* are greatly diverged from the fish collected in Malawi.

4.3.8 The correspondence between analyses with different RAPD primers

In all pair-wise comparisons the correspondence between distance matrices produced by the four RAPD primers was very poor, but significant (Table 4.13). These results compliment those found when estimating genetic differentiation (F_{ST} values) with each primer. The ability of each primer to distinguish between the four wild species from Malawi and the laboratory-bred reference species, can be visualised in dendrograms constructed using neighbour-joining cluster analysis (Figures III.4 to III.7, Appendix III). Cluster analyses of distance matrices produced using all four primers, allowed the clear separation of *O. aureus* and *O. mossambicus* from the four species collected in Malawi. Primer OPA 08, which provided two *O. shiranus* marker fragments, produced the greatest separation of *O. shiranus* from chambo (Figure III.4). All other primers revealed some degree of separation between *O. shiranus* and chambo, although primer OPA 10 which did not generate any *O. shiranus* marker fragments was the least successful. No primers were capable of separating the three chambo species and, although some individuals where grouped together in a similar pattern by different primers, the positions of individual fish generally varied greatly between primers.

Table 4.13 Correspondence between similarity matrices produced by four different RAPD primers based on the correlation coefficient (*r*) (normalized Mantel (1967) statistic *Z*).*

Primers	OPA 08	OPA 10	OPE 15
OPA 10	0.3514		
OPE 15	0.3272	0.3781	
OPG 17	0.3696	0.3081	0.2871

* In all cases the One-tail probability is 0.01 and the *r* value is less than 0.7, indicating a very poor but significant fit. No estimates of *Z* from 100 permutations of the matrices was equal or larger than the observed *Z*.

4.3.9 The correspondence between RAPD and allozyme electrophoretic analyses

The genetic divergence matrices (Nei & Tajima 1981), of 100 individuals that had been used in both RAPD and allozyme analyses, estimated from distance matrices based on only

diagnostic marker fragments and on all RAPD fragments scored, are shown in Table III.4 (Appendix III). The level of correspondence between these two matrices was good (r = 0.84096, P = 0.01). The correspondence between the genetic distance (Nei 1978) matrix estimated from allozymes (Table III.5, Appendix III) and the matrix based on species diagnostic RAPD fragments only was high (r = 0.91205, P = 0.01). However, the correspondence between the allozyme distance matrix and the divergence matrix of all RAPD fragments scored was poor, but still significant (r = 0.73729, P = 0.01).

4.4 DISCUSSION

4.4.1 An appraisal of the RAPD technique

4.4.1.1 Reproducibility of RAPD analyses

An appraisal of the reproducibility of RAPD analysis must consider both the reproducibility of amplification products and the repeatability of scoring. The effects that reaction conditions, such as the magnesium ion concentration and annealing temperature have on the relative intensity of amplified bands has been well documented (Black 1993; Williams *et al.* 1993; Bardakci 1996; Grosberg *et al.* 1996). Here, the brand of *Taq* polymerase used was found to affect the reproducibility of banding patterns. The cause of such variability is uncertain, although it emphasizes how sensitive the amplification procedure is to reaction conditions and composition. The concentration of template DNA also plays a crucial role in the reproducibility of PCR products. The inaccurate quantification of template DNA was the most likely cause of inconsistent results when DNA was pooled, since uneven concentrations of individual DNA samples would allow some DNA templates to have a competitive advantage during amplification. If the concentrations of individual template DNA varies in the repeat analyses, then the banding pattern produced is likely to be different. Indeed, too much template DNA can completely inhibit the amplification reaction (J. Porter *pers. comms.*, University of Wales Swansea). Only the optimization of experimental conditions and the precise use of a chosen protocol will ensure the reproducibility and reliability of RAPD generated banding patterns (Black 1993; Williams *et al.* 1993; Grosberg *et al.* 1996).

The reproducibility of fragment scoring in the preliminary investigation of this study was high (95% reproducibility per lane, 98.6% per gel). Differences in scoring in the preliminary analysis were likely to be attributable to fragments scored as absent in one replication and weakly visible in the second (Heun *et al.* 1994). Therefore, only bands of similar intensity were scored and

great care was taken, by side-by-side gel comparison and estimations of base-pair size, to maximise the probability that fragments scored between gels were homologous. However, in the absence of preliminary pedigree analysis there cannot be complete certainty in the assignment of markers to specific loci (Lynch & Milligan 1994; Grosberg *et al.* 1996). In RAPD analysis, where multiple markers appear on the same gel, there is the possibility that products of different loci will have similar molecular weights and therefore be indistinguishable on a gel. In the present study, extended running times on polyacrylamide gels maximised the separation of fragments. However, despite all efforts to ensure the accurate scoring of co-migrating fragments as homologous, complete confidence in the identity of fragments can only be realised if the markers are isolated and characterised using restriction enzyme analysis (e.g. Furman *et al.* 1997), or their homology verified with DNA hybridization techniques (e.g. Cognato *et al.* 1995; Hilu & Stalker 1995). Southern blots of bands have revealed that even having the same-sized fragment does not necessarily mean that the fragments are homologous (Quiros *et al.* 1995). Furthermore, additional polymorphism can be caused by the presence of heteroduplex molecules formed between allelic RAPD products (Ayliffe *et al.* 1994). It has also been shown that different bands amplified by a single primer can contain homologous sequences, suggesting that different bands are not necessarily independent traits (Smith *et al.* 1994). If the aim of this study was phylogenetic then the complete identification of fragments would be essential. Indeed, several studies have questioned the use of RAPDs for systematics (e.g. Smith *et al.* 1994; van de Zande *et al.* 1995).

4.4.1.2 Analytical value of different primers

In this study, the analytical value of primers varied not only in the number of species-specific markers they provided but also in the power of all fragments scored to detect differentiation between the three species of chambo. Preliminary screening of 23 primers (of disparate sequences) illustrates that some regions of the genome disclose no obvious variation between species, based on the lack of species-specific markers. Unequal rates of evolution between different parts of the genome (Nei 1987) may affect the ability of different primer sequences to reveal differentiation between species (Kambhampati *et al.* 1992). If the employed primer amplifies conserved regions in the genome then these regions are likely to be shared in closely related species, such as the *Oreochromis (Nyasalapia)*, and so little or no variation will be revealed. Estimates of genetic differentiation (F_{ST} values) varied greatly between primers and there was a poor (but significant) correspondence between the distance matrices generated by each primer.

Furthermore, the power of different primers to reveal increased similarity in inbred lines also varied. Observations made in this study compliment those of Bardakci & Skibinski (1994) who found that, although the pattern of similarities and differences between populations showed broad agreement across primers, the overall level of similarity varied between primers. Since individual primers differ significantly in the amount of variation they detect, the present study emphasizes how important it is to analyses a large number of primers, particularly in studies of phylogeny and population genetics. Kambhampati *et al.* (1992) suggested that in order to delineate ancestral relationships among species, at least 20 primers need to be tested.

In an attempt to maximise the analytical value of each primer, when no chambo diagnostic bands were observed, many fragments were scored (average of 40.74 per primer). Generally, far fewer bands are scored per primer in RAPD analysis and the number of primers analysed is usually greater. For example, Bardakci & Skibinski (1994) scored 6 to 17 bands per primer for 13 primers in the identification of tilapia species and subspecies, and Furman *et al.* (1997) scored an average of 1.85 markers per primer for 326 primers in an analyses of genetic relationships between Central American and Mexican pines. Parameters similar to the current study were used in an assessment of genetic variability in wild populations and laboratory strains of the medfly *Ceratitis capitata* (Baruffi *et al.* 1995), where 176 fragments were scored using four primers. Kambhampati *et al.* (1992) emphasized that in general the sample size of primers must be large to quantify the full extent of intraspecific and interspecific variation. Analyses with a large range of primers is far more informative than scoring a large number of fragments across a few primers, since a greater proportion of the genome is screened and the risk of linkage between marker fragments is reduced. However, when the number of primers used is limited (due to resources and time) the information that is contained in fragments that are not conserved in all individuals of a species need not be discarded. In this study, over 25% of fragments scored from primer OPA 08 proved to be valuable in discriminating between chambo species alone. Although not tested, it is likely that the percentage of informative fragments is higher if the farm pond populations and *O. shiranus* are considered. Furthermore, fragments that are unique to individuals are valuable for studies of interpopulation differentiation (Kambhampati *et al.* 1992).

4.4.1.3 The analytical power of RAPD, in comparison to allozyme analysis

RAPD analysis was no more powerful than allozymes in providing diagnostic markers of the three chambo species; *Oreochromis karongae*, *O. lidole* and *O. squamipinnis*. However, since

only 28 of the hundreds of primers available were screened, this study does not rule out the possibility that RAPD could provide diagnostic fragments of the chambo species. The technique has been valuable in highlighting the difficulties of identifying hybrids in populations where extensive backcrossing has occurred. The large number of discrepancies in the identity of farm fish between the two methods of analysis (RAPD and allozymes) does not necessarily mean that these techniques are inefficient, but rather that hybrid backcrossing is so extensive. Therefore, these results emphasize the merits of using more than one type of analysis in this kind of study.

Data generated from the analyses of all 163 RAPD fragments corresponded poorly (but significantly) to that generated by allozymes. High correspondence was observed between allozyme data and RAPD data based on the analyses of diagnostic fragments only. These results suggest that both techniques identified similar frequencies of chambo and *O. shiranus* markers across all populations sampled, whereas the additional information generated by the analyses of non-species-specific RAPD fragments was not seen in the allozyme analysis. Heun *et al.* (1994), in a study of wild oat accessions, found that the two techniques contrast most notably in pair-by-pair comparisons of relationships, and that RAPD analysis resulted in more definitive separation of clusters. Other studies have found that results obtained by RAPD and allozyme electrophoresis correspond well (e.g. Fukuoka *et al.* 1992; Baruffi *et al.* 1995).

Allozyme analysis of the wild populations of *Oreochromis* species in Malawi revealed valuable information concerning the genetic structure of the populations (Chapter 3), whereas RAPD analysis was not so informative. The analysis of population structure using RAPD data is restricted by the lack of complete genotypic information resulting from dominance (Black 1993), firstly because homozygotes cannot be distinguished from heterozygotes, and secondly because a biallelic system cannot be assumed (i.e. there is potential for cryptic variation in the null allelic class). The lack of information greatly reduces the accuracy of allele frequency estimations compared to analysis with co-dominant markers (Lynch & Milligan 1994; Grosberg *et al.* 1996). A number of computer programs are available which can be used to estimate allele frequencies for RAPD data, although these rely heavily on assumptions such as conformation to Hardy-Weinberg equilibrium and linkage equilibrium. Since both of these assumptions are not fulfilled by the populations studied (based on allozyme analysis), it seemed unwise to attempt to transform the data so that more information on population structure could be gained. Furthermore, there was strong evidence to suggest that many loci are not biallelic, based on the relatively high frequency of the null allelic class (i.e. higher frequency of band absence compared to presence), and the high levels

of polymorphism detected with allozymes. Nevertheless, the allozyme distance matrix corresponded well to the RAPD divergence matrix based on species diagnostic RAPD fragments only. However, the correspondence between the allozyme distance matrix and the divergence matrix of all RAPD fragments scored was poorer. It has been estimated that 2 to 10 times more individuals need to be sampled per locus when dominant markers are relied upon to achieve the same degree of statistical power as co-dominant markers (Lynch & Milligan 1994). Therefore, the RAPD technique is best suited to identifying species and for differentiating among conspecific populations.

4.4.2 The genetic status of *Oreochromis* species in the wild and in farm ponds, Malawi

Results obtained by this study largely compliment those found using allozyme analysis, concerning the close relationship between the chambo species, since no markers were found to discriminate between the three species and cluster analysis was unable to separate them. These results are supported by the lack of variation detected by SCAR and SSCP analysis, although it was surprising that no variation was detected between *O. shiranus* and chambo. RAPD analyses did not provide any evidence for the sub-structuring of individual chambo species populations, as suggested in Chapter 3 (Section 3.4.1). The higher frequencies of some RAPD fragments and the occurrence of a unique fragment in the farm pond populations, as compared to wild populations, support the observation of unique alleles in these populations from allozyme analysis (see Chapter 3, Section 3.4.2.1 for discussion of explanations).

The characteristics and incidence of hybridization observed by RAPD analysis largely supports the observations made by allozyme analysis. That is, the incidence of hybridization is largely related to whether any preference for stocking of one species (either chambo or *O. shiranus*) had occurred. Also, the hybrid fish had genotypes that most greatly resembled the species that was chosen for stocking. For example, hybrid fish in the *O. shiranus* pond at Domasi are progeny of a backcross between F_1 hybrids and *O. shiranus,* or of a later generation backcross with *O. shiranus,* as indicated by the occurrence of more *O. shiranus* than chambo diagnostic fragments in all hybrid individuals.

The estimated incidence of hybridization in four of the farm ponds investigated increases if the results of both RAPD and allozymes are combined (assuming that individuals identified as hybrids, by either method, is the correct identity). This increase is most likely to be due to the difficulty in detecting later generation backcross hybrids which have genotypes resembling those

of the parental species. There were no attempts to control breeding in the farm ponds, based on observations made during sampling (Chapter 3, section 3.3.1), and in many ponds fish were breeding. This is particularly true for the ponds at Dwangwa which were densely populated and many females were carrying mouthbroods, and where over 80% of the fish in both ponds (DWSE and DWST) were found to be hybrids. In these conditions it appears that reproductive barriers isolating *O. shiranus* and chambo no longer function because very few of the fish are pure. It is highly likely that in this environment reproductive isolation is not maintained between the chambo species. If those fish identified as pure chambo or *O. shiranus,* in the ponds at Dwangwa, are truly pure than it is possible that these represent individuals which were originally stocked in the ponds or those which have been introduced from the wild during annual restocking.

Although already discussed in the previous chapter, the evolutionary consequences of farm pond escapees of *Oreochromis* species should be emphasized. This study was based on only three farms, but it is highly likely that the results found are representative of many of the tilapia culture ponds in Malawi. The uncontrolled culture of tilapia therefore poses a great risk to the endemic species, from the introduction of both hybrids and cultured fish which may have diverged from their wild conspecifics. However, despite the availability of avenues for escape from farm ponds into the wild, there is no evidence to support this process of potential contamination. Furthermore, the possibility of cultured fish (hybrids or not) surviving in the wild and the extent of their competitive ability, is unknown and requires research.

4.4.3 Further research to identify chambo species

This study has identified the need for a more sensitive technique capable of identifying the very closely related chambo species so that the extent of hybridization in these species can be assessed. Larger sample sizes, in which care is taken to preserve samples immediately, would also be valuable. There are many other RAPD primers which could be screened, although the development of SCAR primers from the chambo-specific fragments identified in this study, and the SSCP analysis of SCAR products, might be valuable in detecting variation between these species. Other molecular techniques are available which have been used successfully in the analyses of tilapia species and sub-species (though these often involve more time consuming protocols than RAPD). For example, Franck *et al.* (1992) sequenced and cloned a family of satellite DNA from species of *Oreochromis*, *Sarotherodon* and *Tilapia* to infer phylogenetic relationships among the tilapiine fishes. Seyoum & Kornfield (1992a, b) used RFLP analyses of mtDNA to

discriminate among subspecies of *O. niloticus*. Preliminary mtDNA analysis revealed fragments, produced using two restriction enzymes, which were diagnostic for *O. lidole* (Turner & Robinson 1991). Mitochondrial DNA analysis alone would be unhelpful in the identification of hybrids, although it would be valuable in detecting the direction of hybridization and in revealing the occurrence of assortative mating when combined with another molecular technique (e.g. Lamb & Avise 1986; Asmussen *et al.* 1989; Scribner & Avise 1994). Finally, the analyses of microsatellite DNA polymorphisms, a technique recently used for the phylogenetic analysis of *Oreochromis* species (including *O. karongae*) and the discrimination of *O. shiranus* subspecies (A. Ambali *pers. comm.*), has great potential for revealing variation between the chambo species.

4.5 SUMMARY

The findings of this study can be summarised as follows:

1) The reproducibility of RAPD amplification products was high, but relies on the precise use of a chosen protocol. The reproducibility of fragment scoring was also high, but relies on the precise scoring of bands of a similar intensity and on side-by-side gel comparisons to maximise the probability that fragments scored between gels are homologous.

2) The analytical value of RAPD primers varied in their ability to detect variation and provide species-specific markers. It is important, particularly in studies of phylogeny and population genetics, to analyse a large number of primers which will provide an adequate representation of the genome.

3) RAPD analysis detected no fixed genetic differences between the chambo species of Lake Malawi, although chambo species can be clearly separated from *O. shiranus* using diagnostic marker fragments. A larger study involving more RAPD primers or the use a more sensitive molecular technique, such as the analyses of microsatellite DNA polymorphisms, may reveal diagnostic markers of these species if indeed they exist.

4) The analysis of population structure using RAPD data is restricted by the lack of complete genomic information. RAPD was able to detect an increase in similarity in inbred lines, and detected some genetic differentiation between chambo species, but overall was no more informative than allozymes.

5) Hybridization between *O. shiranus* and chambo was observed in every farm pond sampled. In a comparison of results obtained from RAPD and allozyme analysis, there were discrepancies in the identity of 40% of fish analysed. These discrepancies were due to the difficulty in identifying

individuals of populations where extensive backcrossing has occurred. In such populations, analyses with two molecular approaches has been valuable. Extensive hybridization has occurred in many of the farm ponds investigated (up to 93.8% hybrids). The incidence and direction of hybridization is largely dictated by the degree to which stocking has been species-specific.

CHAPTER 5

HYBRIDIZATION IN FARM PONDS, MALAWI: EVIDENCE FROM MORPHOMETRIC, MERISTIC AND COLOURATION CHARACTERISTICS

5.1 INTRODUCTION

The previous two chapters have illustrated how molecular genetic techniques can be used to determine the occurrence of hybridization in farm pond populations of Malawi. However, such techniques are often too expensive, specialized and laborious for widespread application by culturists who wish to monitor the identity of their fish stocks. In these circumstances, a reliable method that can be used in the field for quick and easy identification of cultured species, and thereby hybrids, would be valuable. Comparing morphological characters has commonly been used to detect hybridization, particularly before the development of molecular genetic techniques. In this study, the prior identification of hybrids with molecular markers allows the accuracy of using morphological characters to be determined. Furthermore, the morphological, as well as the genetic, characterization of species is required for their conservation and for the effects of cultivation to be determined. Morphological intermediacy is often reported in, and used to identify hybrids (Hubbs 1955; Brown 1995; Wilde & Echelle 1997), although F_1 hybrids often possess unique characters or more closely resembles one of the parental species (McElroy & Kornfield 1993). Where there has been evidence of breeding between hybrids and between hybrids and their parental species, as in this study, intermediacy could either be a direct expression of genetic intermediacy or the sum of the whole spectrum of variants within the sample (Elder *et al.* 1971).

The use of morphometric and meristic characters in the identification of cultured tilapia has been confounded by the overlap of these characters between species (Fryer & Iles 1972; Taniguchi *et al.* 1985). Therefore, more emphasis has often been placed on breeding colouration of the adults in species identification. These characteristics can be misleading taxonomic characters unless the full range of intra-population variation is taken into account. The accurate identification of fish using colouration is also reliant on the specimen being alive or at least very fresh, since colouration can change rapidly after death. Furthermore, many fish may have to be excluded from the analysis if individuals are not in breeding condition or where colouration characteristics are sex-specific. Where morphometric and meristic characters are sufficiently different between the parental species, the identification and characterization of hybrids has been possible (e.g. Elder *et al.* 1971; Galman & Avtalion 1983). Discrimination between tilapiine species has often been improved by the

multivariate analyses of morphometric characters (Pante *et al.* 1988) and discrimination between strains (Velasco *et al.* 1993) and sexes (Brzeski & Doyle 1988) has been achieved. Nevertheless, the majority of studies have suggested that the typical morphometric characters used by taxonomists offer little promise for differentiating tilapia strains and introgressed hybrids (Pante *et al.* 1988; Eknath *et al.* 1991). The identification of species based on morphological characters is also complicated by the potential of such characters to be affected by environmental factors (Ihssen *et al.* 1981; Purdom 1993). This is particularly true in the culture of tilapia where the widespread distribution of many species has often resulted in fish being reared under different environmental conditions to which they were originally described (McAndrew & Majumdar 1983). The morphological differentiation of cultured stocks, due to genetic and environmental influences, must be considered before characteristics distinguishing hybrids of cultured species from pure wild species can be identified.

The morphological characteristics of the *Oreochromis* species of Lake Malawi have been studied to various degrees by a number of authors, although there have been few accounts of these species in aquaculture and no descriptions of hybrids between *O. shiranus* and the *Oreochromis (Nyasalapia)* (chambo). *O. shiranus* can be clearly distinguished from chambo by a number of characteristics (see Table 3.1, Chapter 3), whereas the meristic and morphometric characteristics of chambo broadly overlap (Trewavas 1983). Trewavas (1941) distinguished *O. karongae* and *O. lidole* largely on the basis of dentition and the shape of pharyngeal bone, although these characters also overlap. Catch statistics recorded by the Malawi fisheries department failed to distinguish species (Alimoso 1986; cited in Turner *et al.* 1989b) and field observations of body colouration and nesting behaviour by McKaye & Stauffer (1988) also failed to identify species reliably. An extensive morphometric study conducted by Turner *et al.* (1989b) involved the analyses of 23 measurements of chambo species held at the British Museum of Natural History (BMNH). Species discrimination was found to be a multivariate process dependent on the inter-relationship of many separate measurements and no single measurement was entirely diagnostic of any species. In a later study (Turner & Robinson 1991), it was realised that specimens collected in the field were different from the described species (based on samples held at the BMNH) due to habitat-related geographic variation, particularly in *O. karongae.*

The aims of this study, using morphological analysis, were:

1) To determine whether it was possible to distinguish between *O. shiranus* and chambo, using a limited number of morphometric and meristic characters that are quick and easy to record in the

field.

2) To determine if it was possible to distinguish between species of chambo using morphometric and meristic characters. And to identify the unknown cases of chambo, collected in the wild, using some or at least all of the morphological characters recorded (i.e. morphometric, meristic and colouration).

3) To determine if hybrids can be discriminated from pure species, and therefore identified using a limited number of morphometric and meristic characters. The reliability of this method can be tested by the prior identification of hybrids with genetic analyses.

4) To ascertain how reliable and useful colouration is in the identification of hybrids, based on prior identification with genetic techniques, and determine if the identification is different from that found with morphometric and meristic characters.

5.2 METHOD

5.2.1 Sampling and data recorded

Details of sample sites are given in Chapter 3 (Figure 3.5 and Table 3.2). Morphological details were recorded for all the pure fish sampled from the wild where tissue was taken for allozyme analyses. Morphological details were taken for 70 fish from five different farm ponds. All farm pond fish identified as hybrids using allozyme (Chapter 3) or RAPD analyses (Chapter 4), where morphological details were recorded, will be used as representatives of hybrid individuals. For convenience, samples of pure species and hybrids will be referred to as 'groups'. Details of the number of fish analysed, of both sexes, are given in Table 5.1.

The morphometric characters recorded were selected on their diagnostic

Table 5.1 The number of pure (upper table) and hybrid (lower table) fish, of each sex, used for morphological analyses. Details for hybrids are further broken down into the farm pond of origin. The total number of farm fish where morphological details were recorded (including hybrid and 'pure' fish) are shown in parenthesis.

Group*	no. of males	no. of females	Total
OSHI	16	5	21
OKAR	13	11	24
OLID	13	5	18
OSQU	23	0	23
HYBRIDS	42	9	51
DMOK	2 (10)	0 (0)	2 (10)
DMSH	9 (10)	0 (0)	9 (10)
DWSE	9 (11)	4 (4)	13 (15)
DWST	19 (21)	4 (4)	23 (25)
MZKH	3 (7)	1 (3)	4 (10)

* OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; HYBRIDS, all hybrids detected in the five farm ponds; DMOK, *O. karongae* pond Domasi; DMSH, *O. shiranus* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storage tank pond; MZKH, *O. karongae* holding pond Mzuzu.

value of discriminating *O. shiranus* and chambo (George Turner *pers. comm.*) and for their ease of recording in the field. Eight measurements were made with dial callipers (to the nearest 0.1 cm), and standard length was measured using a ruler (Table 5.2). Morphological characters used to identify the four wild species were also recorded. These include the number of anal fin spines, number of teeth rows, teeth row arrangement and colouration. Teeth row arrangement was classified to one of three types: 1) first row distinct and following rows overlapping; 2) all rows distinct; and 3) all rows overlapping and indistinct. Details of colouration were recorded only for specimens that were alive or extremely fresh. The colouration characteristics recorded, that would be helpful in identifying species (see Table 3.1, Chapter 3), included the colour of the body (excluding the head and fins), caudal fin margin, dorsal fin margin and the presence of a face mask. The presence of vertical bars or horizontal stripes can be useful characters in discriminating between chambo and *O. shiranus,* and were therefore recorded. The presence of a genital tassel was noted and the sex of each fish was determined by internal examination. All meristic and colouration characters were coded into 'dummy' variables, represented by 1 for present and 0 for absent.

Table 5.2 Descriptions and abbreviations (Abbr.) of nine measurements taken during sampling of wild and farm fish in Malawi (adapted from Turner *et al.* 1989b).

Measurement	Abbr.	Description
Standard length	S.L.	- anterior end of upper jaw to base of caudal fin
Maximum body depth	B.D.	- maximum overall body depth (exclusive of fins)
Caudal peduncle depth	C.D.	- depth of caudal peduncle measured at the last ray of dorsal or anal fin (whichever is most posterior)
Caudal peduncle length	C.L.	- posterior end of base of anal fin, to point on the line of flexion of hypurals at which is inserted the most ventral caudal which reaches to posterior margin of caudal fin
Head length	H.L.	- anterior end of upper jaw to the most posterior part of bony operculum
Interorbital width	I.W.	- minimum bony interorbital distance, measured superior to the angle of the jaw
Lower jaw length	L.J.	- anterior end of lower jaw to posterior end of the jaw above the retroarticular and not the posterior of the lip
Maximum body width	M.W.	- maximum overall body width (usually over operculum)
Snout length	SN.L.	- length from the anterior of upper jaw to the foremost part of the orbit

5.2.2 Data analyses

5.2.2.1 Size adjustment and the morphometric analyses of Oreochromis *species*

To test for differences in the body size of fish between groups, pair-wise comparisons were made on the standard length (original measurement) using the Mann-Whitney test. Measurements were corrected for size to ensure that any differences detected between groups were in terms of shape rather than size. There are a large number of methods used in the literature for size-adjustment, each with their own particular merits and downfalls (see Thorpe & Leamy 1983; Reist 1985; Rohlf & Bookstein 1987, for relevant discussions). Therefore, three methods of size-adjustment were selected, and tested on the data of the pure species only. The three methods selected (ratios and two allometric adjustments) are relatively simple univariate methods (Reist 1985). Despite the tendency of ratios to cause non-randomly distributed variances in the data, this method was chosen because it does not require complex computation and the other methods of size adjustment are often too complicated for the field. The two allometric methods were chosen because they are also relatively simple, and because they have been found to effectively adjust for size without losing shape variation (Reist 1985; Hauser 1996).

The three methods used are as follows:

1) RATIO $M_2 = M_1 / S.L.$

2) ALLOM1 $M_2 = \log M_1 - \beta_1 (\log S.L. - \log S.L._{avg})$

3) ALLOM2 $M_2 = \log M_1 - \beta_2 (\log S.L. - \log S.L._{avg})$

Where: M_1 = original measurement
M_2 = size adjusted measurement
S.L. = standard length
$S.L._{avg}$ = overall mean of standard length
β_1 = slope of overall regression of log M_1 against log S.L.
β_2 = pooled within-sample slope of regression of log M_1 against log S.L.

The significance of correlations between size-adjusted measurements and S.L. were tested to assess how effective each method was at removing the effects of size. Methods of size-adjustment were further tested by submitting the transformed data to principal component analysis (PCA) and discriminant function analysis (DFA) to determine which method retained the greatest amount of 'shape' variation and was therefore most effective at discriminating between groups. PCA of size-related data typically produces high and positive loadings on the first component (Thorpe 1976). In PCA linear combinations of the observed variables are formed, so that the variation associated with each of a number of variables are summarised into a smaller number of

principal components without the prior assignment of individuals to groups. Correlations between the original variables and the principal components (i.e. component loadings) were used to interpret the importance of individual measurements in the description of the variation of the data set. Plots of PCA scores from the first two principal components, which explain the greatest amount of variation in the sample set, were made to determine the amount of separation achieved among species using morphometric characters. The amount of separation achieved with data size-adjusted with different methods were compared.

Discriminant function analysis (DFA) combines the scores of all variables into discriminant functions in such a way as to maximize separation into the predetermined groups, and therefore requires *a priori* classification of individuals into groups being discriminated. Linear combinations of the independent variables are formed and serve as the basis for reclassifying cases into one of the groups. The percentage of correctly classified individuals were used to measure the morphological distinctness between species (based on the eight measurements), and to determine which method of size adjustment allowed the greatest distinction. Discriminant functions can be interpreted in a similar way to component loadings in a PCA, and were therefore used to identify variables most useful in distinguishing among groups. Plots of case scores from the first two discriminant functions, and of group centroids, were made to visualise the amount of separation between groups, and the relative positions of misclassified cases. The significance of discriminant functions, that is, whether discriminant functions reflect true group differences and not sampling variability, was tested as part of the DFA using Wilks' lambda. The significance level of the observed Wilks' lambda was based on a chi-square transformation of the statistic. All analysis was carried out using the SPSS computer package for windows, and the analysis described above was used when data were submitted to a PCA and DFA.

5.2.2.2 Univariate analysis of morphological characters in pure and hybrid fish

Size-adjusted measurements were initially tested for significant differences between sexes by analysis of variance between males and females for all groups (pure species and hybrids). One group (*O. squamipinnis*) did not contain females, and the number of females sampled was generally small due to the characters originally used to identify species during sampling. The small sample sizes of females may not be representative of this sex and could induce spurious results. To prevent exclusion of females from the analyses, two tests were conducted: one between males only, and the other between all fish (males and females). Furthermore, it is valuable to establish

the amount of discrimination which can be achieved between groups containing both sexes, since accurate sexing requires the dissection and therefore death of individuals which may not be desired. Moreover, it may not be possible to sex immature fish.

Size-adjusted measurements were analysed individually using analysis of variance between the groups, using the Bonferroni procedure to determine which groups were significantly different. Analyses were carried out on different sets of hybrids. First, on a single group containing all hybrids (from five farm ponds); and second, on two groups of hybrids from only the Dwangwa sewage pond (DWSE) and the Dwangwa storage pond (DWST). Hybrids of the remaining farm ponds (DMOK, DMSH and MZKH) were not analysed separately because group sizes were very small. Both analysis also included the groups of pure species (OSHI, OKAR, OLID and OSQU).

The morphological variability within groups was assessed by estimating the coefficient of variations (CV) at each size-adjusted measurement. Difference in morphological variability between groups was tested by Wilcoxon signed rank tests. As before, hybrids were represented by a single group and by two separate groups from two different farm ponds (DWSE and DWST).

Difference in teeth row count and teeth row arrangement between groups was tested by Mann-Whitney tests. There were four missing hybrids in this analysis, because the teeth rows were too small to be counted. Hybrids were represented by a single group and by two separate groups from two different farm ponds (DWSE and DWST).

5.2.2.3 Multivariate analysis of morphological characters in wild, farm and hybrid fish

The data of size-adjusted measurements, teeth row count and arrangement, and anal fin spine count were submitted to the multivariate analysis (PCA and DFA) of all groups (with hybrids as a single group). A second DFA was conducted using only the groups of pure species. The discriminant scores were then used to classify the 'ungrouped' hybrid cases, to determine which species individual hybrids most resemble based on morphological characters. All tests were conducted on males alone and on both sexes together. A further DFA was performed to determine if cultured fish from two different ponds (DMOK where hybrids are rare, and DMSH where hybrids are common) could be distinguished from each other and from wild fish. This analyses was conducted in order to determine whether any prior discrimination of hybrid fish from wild fish was due to morphological differences between hybrid and wild fish, and not due to differences between cultured and wild fish. This analyses was conducted on males only, since DMOK and DMSH farm pond samples did not contained females.

5.2.2.4 Analyses of colouration in wild fish and the identification of hybrids

In the analyses of colouration data, the percentage of cases in each group possessing each character was determined. All variables recorded (morphometric, dentition and colouration) were used in a DFA of the pure species. The discriminant scores were then used to classify eleven ungrouped individuals of unknown identity that were collected in the wild. The discrimination of hybrids from pure species would probably be improved if variables of colouration are included, but it was decided that this may be misleading. This decision was made on the basis that the absence of a particularly character in a farm fish (which could result in discrimination from pure species) may be due to its sex, breeding condition and maturity, rather than its identity. However, the number of individuals that could be identified as putative hybrids based on species-specific colouration alone was counted. In a repeat of this analysis, anal fin spine count was also taken into account to determine how reliable fish identification was based on colouration alone.

5.3 RESULTS

5.3.1 Size adjustment and the morphometric analysis of *Oreochromis* species

The need for the size adjustment of measurements was exemplified by the large range of standard lengths (S.L.) within groups, and the significant difference ($P<0.05$) found in standard lengths between all groups analysed (Table 5.3). The standard length of *O. lidole* was greater than all other groups, whereas the farm pond hybrids were far smaller than the groups of wild fish.

Table 5.3 Mean, standard deviation and range of standard length (S.L.) in pure species and hybrids and results of Mann-Whitney tests (U-statistic and significance) of S.L. between groups.

Group	no. of cases	Mean ± Std.	Range	U-statistic and Significance			
				OSHI	OKAR	OLID	OSQU
OSHI	21	192.0 ± 20.5	(136.0 - 229.0)				
OKAR	24	228.2 ± 32.8	(187.0 - 312.0)	69.0 ***			
OLID	18	274.9 ± 21.7	(231.0 - 318.0)	0.0 ***	52.5 ***		
OSQU	23	208.1 ± 17.4	(184.0 - 251.0)	136.0 *	165.0 *	4.0 ***	
HYBRID	51	128.3 ± 23.9	(78.0 - 184.0)	29.5 ***	0.0 ***	0.0 ***	0.5 ***

OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; *$P<0.05$; ***$P<0.001$.

When measurements were adjusted for size using the RATIO method, the influence of size variation was not removed from the data; half of the size-adjusted measurements were still correlated with S.L. (Table 5.4). ALLOM1 appeared to remove all the size effects, as none of the

regressions with S.L. were significant. Whereas, five of the eight regressions between ALLOM2 adjusted measurements and S.L. were significant. However, this was expected as the pooled within-group slope was used to adjust for size, whereas the regressions tested the significance of the overall slope (Hauser 1996). Therefore, both ALLOM1 and ALLOM2 methods were further tested with PCA and DFA.

Table 5.4 Efficiency of size adjustment of different transformation methods. Regressions between S.L. and each of the size adjusted measurements* were tested for significance.

	RATIO		ALLOM1		ALLOM2	
	r^2	sign.	r^2	sign.	r^2	sign.
B.D.	0.001	n.s	<0.001	n.s	0.071	***
C.D.	0.349	***	<0.001	n.s	0.092	***
C.L.	0.371	***	<0.001	n.s	0.001	n.s
H.L.	0.012	n.s	0.004	n.s	0.001	n.s
I.W.	0.674	***	<0.001	n.s	0.038	*
L.J.	0.006	n.s	0.003	n.s	0.030	*
M.W.	0.012	n.s	0.001	n.s	0.141	***
SN.L.	0.626	***	0.002	n.s	0.001	n.s

** see Table 5.2 for description of measurements; n.s, not significant; *P<0.05; ***P<0.001.

Three principal components (PCs) were extracted from the data, size adjusted by the two methods (Table 5.5). loadings of the first PCs were similar for both methods: explaining approximately 40% of the total variance, with the highest loadings on variables associated with head shape (H.L., SN.L. and I.W.). Loadings were variable in size and magnitude, which suggests that size was removed effectively from the data by both methods. The first and second PCs of ALLOM2 explained slightly more of the variance than those of ALLOM1 (67.7% compared to 66.4%). The PCA plots (Figure 5.1)

Table 5.5 Loadings, and percentage of variance explained for the three principal components (PC) extracted by PCA on morphometric data*, of the four wild pure species (males and females), size adjusted by two different methods (ALLOM1 and ALLOM2).

	ALLOM1			ALLOM2		
	PC 1	PC 2	PC 3	PC 1	PC 2	PC 3
B.D.	-0.111	0.284	0.901	0.039	0.374	0.880
C.D.	-0.352	0.802	0.342	-0.227	0.883	0.243
C.L.	0.493	-0.730	0.250	0.496	-0.741	0.193
H.L.	0.927	0.199	-0.112	0.927	0.171	-0.126
I.W.	0.764	-0.439	0.337	0.783	-0.463	0.256
L.J.	0.575	0.629	-0.418	0.541	0.609	-0.486
M.W.	0.614	0.230	0.379	0.648	0.238	0.297
SN.L.	0.831	0.351	-0.058	0.826	0.331	-0.106
% var.	40.3	26.1	18.0	39.6	28.1	16.1

* see Table 5.2 for description of measurements; % var., Percentage of variance explained.

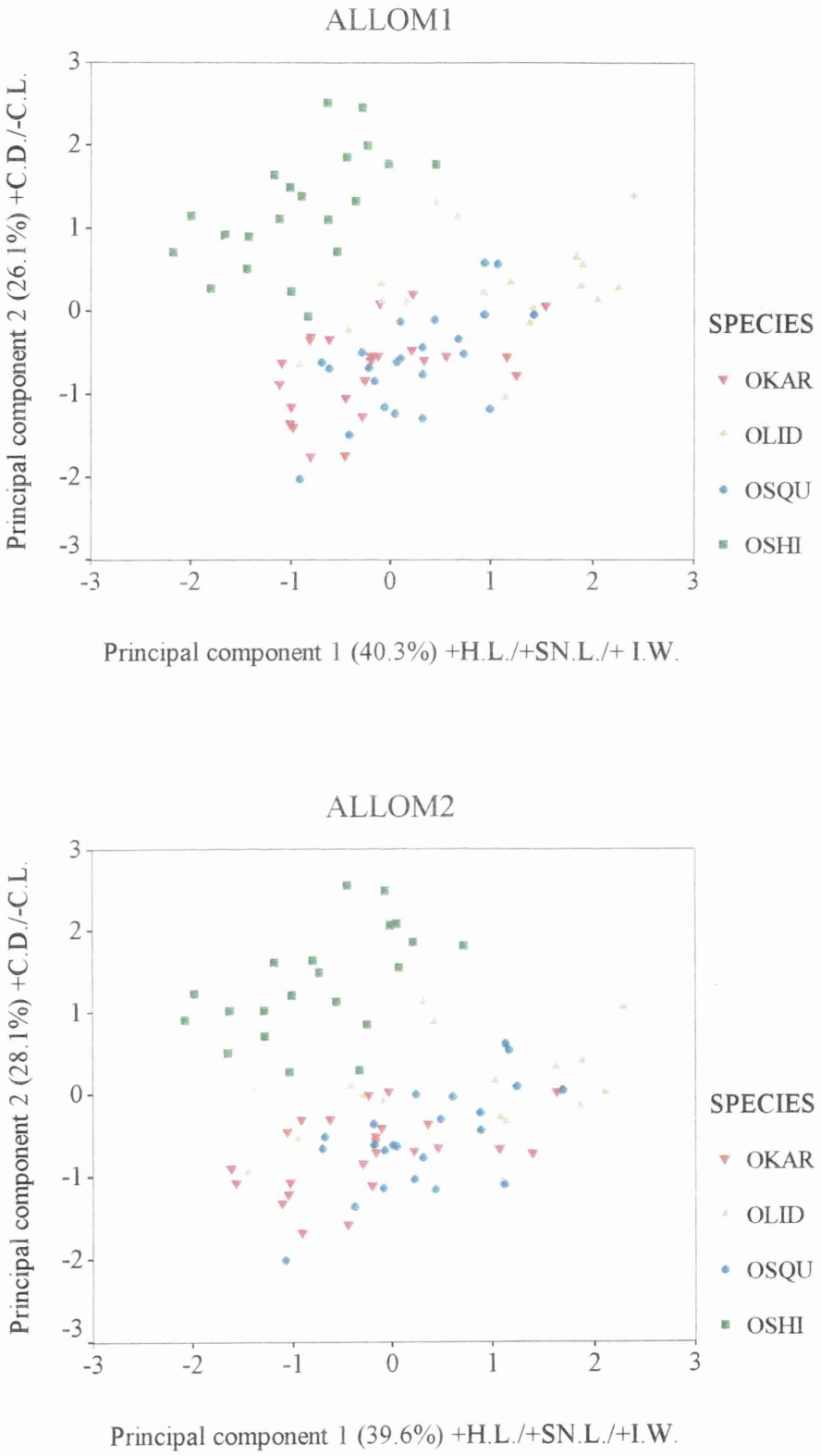


Figure 5.1 Plots of first and second PC scores of a PCA using data, of the four pure species (males and females) (OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis;* OSHI, *O. shiranus*) sampled from Lakes Malawi and Malombe, transformed with two size adjustment methods (ALLOM1 and ALLOM2). Variables most correlated with the respective PCs are shown on the axis, with their sign of correlation.

indicated that both methods separated *O. shiranus* from chambo on the second PC, which was highly correlated with caudal fin shape (C.D. and C.L.). However, the separation of chambo species was poor for both methods. It was not clear which method was most effective at retaining shape variation so the data were submitted to DFA.

Plots of the first and second discriminant functions (DF), from the DFA of data size adjusted by the two methods, showed better separation of chambo species than that shown in the PCA plots, although there was still some overlap between *O. squamipinnis* and *O. karongae* (Figure 5.2). For both size adjustment methods, all DFs explained some of the variance between groups (Wilks' lambda; $P<0.05$), although the amount of variance explained by the first two DFs was slightly greater for ALLOM2 than ALLOM1 (97.2% compared to 95.6%) (Table 5.6). *O. shiranus* and chambo species were separated on the first DF, which was highly correlated with caudal fin measurements (C.D., C.L.); over 95% of *O. shiranus* cases were correctly classified. The separation of *O. lidole* from *O. karongae* and *O. squamipinnis* was largely on the second DF, which was highly correlated with variables associated with head shape (H.L., L.J. and SN.L.). The number of individuals classified correctly, over all groups, was slightly higher for data size-adjusted by the ALLOM2 method (83.7% compared to 80.23%) (Table 5.7). The ALLOM2 method was, therefore, used for further analyses of the total data set.

Table 5.6 Within-groups correlations for each measurement*, percentage of variance explained and the significance of functions (Wilks' Lambda) for the three canonical discriminant functions obtained from DFA on morphometric data size adjusted by two different methods, for males and females of the four pure species sampled in Malawi.

	ALLOM1			ALLOM2		
	Function 1	Function 2	Function 3	Function 1	Function 2	Function 3
B.D.	-0.041	-0.403	0.795	-0.019	-0.454	0.602
C.D.	-0.572	-0.310	0.436	0.552	0.271	-0.031
C.L.	0.517	0.009	-0.036	-0.510	-0.365	0.152
H.L.	0.151	0.617	0.389	0.067	0.525	0.491
I.W.	0.469	0.153	0.083	0.460	0.338	0.123
L.J.	-0.206	0.839	0.260	-0.276	0.806	0.295
M.W.	-0.051	0.154	0.248	0.040	0.203	0.205
SN.L.	-0.036	0.529	0.227	-0.022	0.461	0.295
% var.	76.49	20.75	2.76	82.88	12.73	4.39
Wilks'	0.063 ***	0.368 ***	0.851 *	0.071 ***	0.437 ***	0.785 **

* see Table 5.2 for description of measurements; % var., Percentage of variance explained; Wilks', Wilks' Lambda and it's significance for current and remaining functions, where *** $P<0.001$; *$P<0.05$ and n.s, not significant.

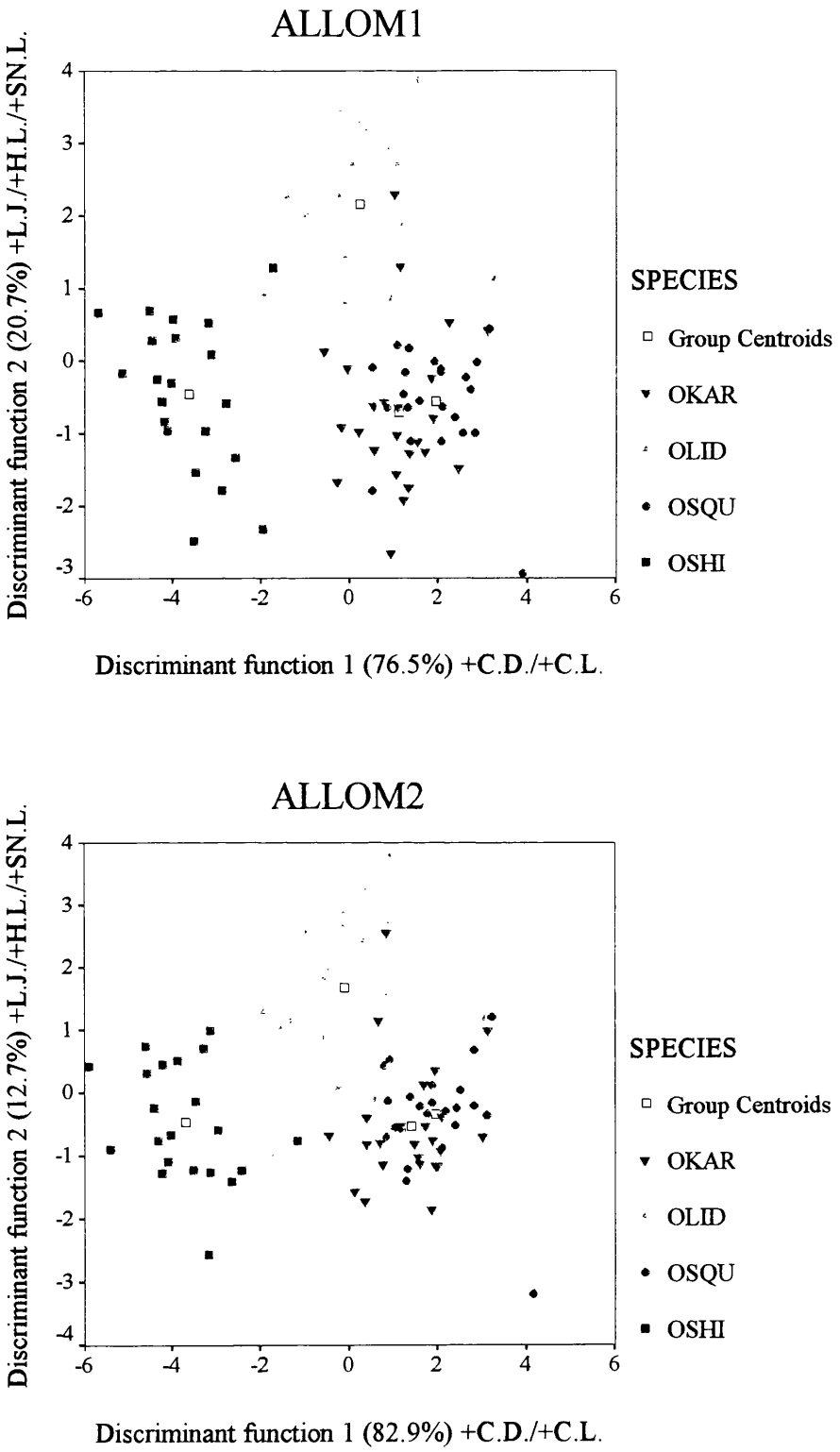


Figure 5.2 Plots of first and second discriminant function scores of a DFA using morphometric data transformed with two size adjustment methods (ALLOM1 and ALLOM2) for males and females of the four pure species (OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis;* OSHI, *O. shiranus*) sampled from Lakes Malawi and Malombe. Variables most correlated with the respective DFs are shown on the axis, with their sign of correlation.

Table 5.7 Classification results obtained for DFA on morphometric data, size adjusted by two different methods, for both sexes of the four pure species sampled in Malawi. Percentage of cases correctly classified is 80.23% for ALLOM1 and 83.7% for ALLOM2.

Actual group*	no. of cases	Predicted group membership - percentage of cases							
		OSHI		OKAR		OLID		OSQU	
		ALLOM1	ALLOM2	ALLOM1	ALLOM2	ALLOM1	ALLOM2	ALLOM1	ALLOM2
OSHI	21	95.2	95.2	0.0	4.8	4.8	0.0	0.0	0.0
OKAR	24	0.0	0.0	62.5	66.7	8.3	8.3	29.2	25.0
OLID	18	11.1	5.6	0.0	0.0	83.3	83.3	5.6	11.1
OSQU	23	0.0	0.0	17.4	8.7	0.0	0.0	82.6	91.3

* OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*.

5.3.2 Univariate analysis of size-adjusted measurements

The mean, standard deviation and range of each measurement recorded for males only, and for males and females together are given in Table IV.1, Appendix IV. Data for hybrid fish are shown for all hybrids together (from five farm ponds), and for hybrids from just the DWSE and DWST ponds. No significant differences were detected between measurements of males and females for *O. shiranus* and *O. karongae*. In contrast, five of the eight measurements were significantly different ($P<0.05$) between sexes in *O. lidole,* and SN.L. was significantly different ($P<0.005$) between sexes in hybrids. SN.L. was found to differ significantly ($P<0.001$) between sexes across all groups (Table 5.8).

Table 5.8 Analysis of variance (F statistic & significance) between males and females for each size -adjusted measurement*, for pure species (except *O. squamipinnis* where only males were sampled), hybrid fish, among all pure species and among all the populations sampled.

	O. shiranus	*O. karongae*	*O. lidole*	Among pure spp.	Hybrid	Among all
no. ♂	16	13	13	42	42	84
no. ♀	5	11	5	21	9	30
B.D.	3.60 n.s	2.43 n.s	0.54 n.s	0.60 n.s	0.51 n.s	0.11 n.s
C.D.	1.68 n.s	1.57 n.s	0.10 n.s	0.01 n.s	0.07 n.s	0.45 n.s
C.L.	0.36 n.s	0.09 n.s	5.42 *	1.05 n.s	0.67 n.s	0.27 n.s
H.L.	1.52 n.s	0.00 n.s	6.64 *	2.24 n.s	0.84 n.s	2.22 n.s
I.W.	0.00 n.s	0.28 n.s	5.01 *	1.22 n.s	0.38 n.s	0.83 n.s
L.J.	0.16 n.s	0.07 n.s	7.85 *	1.31 n.s	1.82 n.s	2.40 n.s
M.W.	0.13 n.s	0.01 n.s	0.71 n.s	1.12 n.s	1.53 n.s	0.01 n.s
SN.L.	2.96 n.s	0.29 n.s	6.32 *	6.75 *	9.59 **	15.20 ***

* see Table 5.2 for description of measurements; no. ♂, number of males; no. ♀, number of females; n.s, not significant; *$P<0.05$; **$P<0.005$; ***$P<0.001$.

All of the size-adjusted measurements, except maximum body width (M.W.), produced significant differences between groups (Table 5.9). There was little difference in results between the tests on males only and on males and females together. *O. shiranus* was characterised from all other groups by the following features: deeper caudal peduncle; shorter caudal peduncle; shorter interorbital width. *O. lidole* was characterised by the following features: shallower body (than *O. squamipinnis*); longer head (than *O. karongae* (when males tested only) and *O. squamipinnis*); longer lower jaw (than all groups); longer snout (than *O. karongae*). No significant differences where detected in measurements between *O. karongae* and *O. squamipinnis*.

Table 5.9 Analysis of variance (F-statistic and significance) on size-adjusted measurements* of all groups (pure species and hybrids); tested on males only and on males and females together. The Bonferroni procedure was used to determine which groups were significantly different at the 5% level. The direction of difference (more than, or less than) between groups is indicated.

	Males only			Males and females		
	F	*P*	Groups differing at $P<0.05$	F	*P*	Groups differing at $P<0.05$
B.D.	11.4	***	HYBRID < OSHI & OSQU OLID < OSQU	12.3	***	HYBRID < OSHI, OKAR, & OSQU OLID < OSQU
C.D.	18.7	***	OSHI > OKAR, OLID, OSQU & HYBRID	21.9	***	OSHI > OKAR, OLID, OSQU & HYBRID
C.L.	14.5	***	OSHI < OKAR, OLID, OSQU & HYBRID OSQU > HYBRID	19.4	***	OSHI < OKAR, OLID, OSQU & HYBRID OSQU > HYBRID
H.L.	11.3	***	OSHI < OLID & HYBRID OKAR < OLID & HYBRID OLID > OSQU	10.0	***	OSHI < OLID & HYBRID OKAR < OLID & HYBRID
I.W.	18.7	***	OSHI < OKAR, OLID, OSQU & HYBRID HYBRID < OKAR, OLID & OSQU	20.9	***	OSHI < OKAR, OLID, OSQU & HYBRID HYBRID < OKAR, OLID & OSQU
L.J.	11.7	***	OLID > OSHI, OKAR, OSQU & HYBRID HYBRID > OKAR & OSQU	12.9	***	OSHI > OKAR OLID > OSHI, OKAR, OSQU & HYBRID HYBRID > OKAR & OSQU
M.W.	0.8	n.s		1.0	n.s	
SN.L.	5.4	**	OKAR < OLID & HYBRID HYBRID > OSQU	4.6	**	OKAR < OLID & HYBRID

* see Table 5.2 for description of measurements; n.s, not significant; $**P<0.005$; $***P<0.001$.
OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*

The hybrid group was characterised by the following features: shallower body (than *O. shiranus*, *O. karongae* (when males and females tested only) and *O. squamipinnis*); shorter interorbital width (than chambo species); longer lower jaw (than *O. karongae* and *O. squamipinnis*). These observations, based on hybrids from different farm ponds, did not generally hold true when the hybrids from two farm ponds were analysed separately (Table 5.10). For

example, hybrids of both ponds did not have a significantly shallower body depth than *O. karongae*; hybrids of the DWSE pond only, had a shorter interorbital width than chambo species; hybrids of the DWST pond only, had a longer lower jaw than *O. squamipinnis*. The results of tests based on males only and on both sexes together, were often different. Significant differences in measurements between DWSE and DWST hybrids, were also observed; caudal length was greater in DWSE (when both sexes tested only); whereas, head length and snout length were greater in DWST. In general, the least number of significant differences were found between the measurements of DWST and *O. lidole*, and between DWSE and the two groups *O. shiranus* and *O. karongae*. During sampling it was noted that there was great variation in the body shape, particularly the head shape, of fish sampled from the sewage pond and storage pond at Dwangwa. Fish had a stunted appearance, with a large head which was accentuated by large eyes. The snout shape varied from almost pointed (indicative of *O. shiranus*) to convex and rounded (indicative of chambo) (Figure 5.3).

Table 5.10 Analysis of variance (F-statistic and significance) on size-adjusted measurements*; as in Table 5.9, but the HYBRID group (containing all hybrids) is replaced with two separate groups of hybrids from the farm ponds DWSE and DWST. Only those groups which are significantly different from hybrids of the two farm ponds are shown.

	Males only			Males and females		
	F	*P*	Groups differing at $P<0.05$	F	*P*	Groups differing at $P<0.05$
B.D.	5.1	***	DWST < OSQU & OSHI	4.7	***	OSQU > DWSE & DWST
C.D.	17.2	***	OSHI > DWSE & DWST	19.5	***	DWSE > DWST
C.L.	20.1	***	OSQU > DWSE DWST < OKAR, OLID & OSQU	21.4	***	OSHI < DWSE & DWST OSQU > DWSE & DWST
H.L.	24.0	***	DWSE < OLID & OSQU DWST > OSHI, OKAR & OSQU	25.0	***	DWSE < OLID & OSQU DWST > OSHI, OKAR & OSQU
I.W.	24.4	***	OSHI < DWST DWSE < OKAR, OLID & OSQU	29.4	***	OSHI < DWST DWSE < OKAR, OLID & OSQU
L.J.	14.7	***	DWST > OKAR & OSQU	17.1	***	DWSE > OKAR, DWSE < OLID DWST > OKAR & OSQU
M.W.	1.3	n.s		2.3	n.s	
SN.L.	9.8	***	DWST > OSHI, OKAR & OSQU OLID > DWSE, DWSE < DWST	10.5	***	DWST > OSHI, OSQU & OKAR OLID > DWSE, DWSE < DWST

* see Table 5.2 for description of measurements; n.s, not significant; **$P<0.005$; ***$P<0.001$;
OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; DWSE, Sewage pond Dwangwa; DWST, Storage pond Dwangwa.

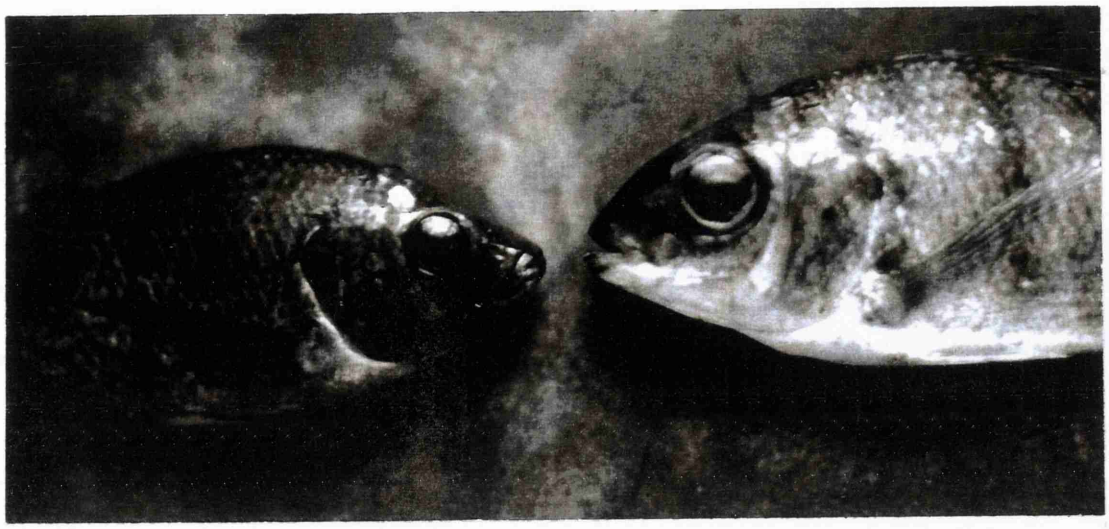


Figure 5.3 Two fish from the sewage pond at Dwangwa, showing the large eye size and stunted appearance observed in most fish sampled, and the variety of snout shapes: pointed (fish on left) and rounded (fish on right) (Photograph by Eric Roderick).

5.3.3 Morphological variability

In most cases, more variation was observed in each size-adjusted measurement when both sexes were grouped (Table 5.11). Coefficients of variation (CV), across all measurements, were significantly greater in the hybrid group, when compared to all groups of pure species. The CVs of hybrids in two separate farm ponds (DWSE and DWST) were also greater than all groups of pure species, but only when males were analysed alone (Table 5.12). Significant differences in the

Table 5.11 Coefficient of variation (CV) of each size-adjusted measurement* for male only, and male and female pure and hybrid fish.

	OSHI		OKAR		OLID		OSQU	HYBRID		DWSE		DWST	
	♂	♂ & ♀	♂	♂ &♀	♂	♂ & ♀	♂	♂	♂ & ♀	♂	♂ & ♀	♂	♂ & ♀
B.D.	1.00	1.33	1.12	1.17	0.67	1.02	0.98	1.59	1.54	1.59	1.59	1.21	1.18
C.D.	1.17	1.58	0.99	1.12	0.96	1.48	1.19	2.61	2.49	1.83	1.57	2.48	2.35
C.L.	1.38	1.83	2.26	2.32	2.17	2.52	1.58	3.27	3.05	2.72	2.38	2.43	2.77
H.L.	0.65	0.82	0.86	0.87	0.75	0.98	0.66	1.24	1.28	0.67	0.58	0.95	0.88
I.W.	1.92	1.84	1.68	1.54	1.54	2.24	1.11	2.67	2.71	1.22	1.21	2.63	2.62
L.J.	2.08	2.44	2.47	2.14	1.67	2.56	2.24	4.44	4.52	3.65	3.41	3.79	3.82
M.W.	1.66	1.75	1.51	1.73	1.62	1.68	1.44	2.06	2.06	2.63	2.53	1.51	1.44
SN.L.	1.13	1.43	1.74	1.80	1.26	1.66	1.38	2.75	2.90	2.21	2.07	2.28	2.31

* see Table 5.2 for description of measurements; OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; DWSE, Sewage pond Dwangwa; DWST, Storage pond Dwangwa.♂, males only; ♂ & ♀, males and females; Std., Standard deviation of mean CV.

CVs of pure species where found only between males of *O. karongae* and *O. lidole,* and between males of *O. karongae* and *O. squamipinnis*; the CV of male *O. karongae* were generally greater.

Table 5.12 Comparisons of CVs (across all measurements) between pure and all hybrid fish, and between pure and hybrid fish from DWSE and DWST by a Wilcoxon signed ranks test (test statistic and significance), for males only and for both sexes together.

	OSHI		OKAR		OLID		OSQU	
Group	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀
OKAR	-1.12 n.s	-0.28 n.s						
OLID	-0.70 n.s	-1.26 n.s	-2.10 *	-1.40 n.s				
OSQU	-0.14 n.s	-	-2.10 *	-	0.00 n.s	-		
HYBRID	-2.52 *	-2.52 *	-2.52 *	-2.52 *	-2.52 *	-2.52 *	-2.52 *	-2.52 *
DWSE	-1.96 *	-1.40 n.s	-2.03 *	-1.54 n.s	-2.10 *	-0.70 n.s	-2.52 *	-2.38 *
DWST	-2.38 *	-1.82 n.s	-2.37 *	-2.10 *	-2.38 *	-1.96 *	-2.52 *	-2.37 *

OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; DWSE, Sewage pond Dwangwa; DWST, Storage pond Dwangwa; ♂, males only; ♂ & ♀, males and females; n.s, not significant; *P<0.05.
-, comparison not made because the *O. squamipinnis* sample did not contain females.

5.3.4 The analysis of teeth row count and arrangement in pure species and hybrids

No significance differences were found in teeth row count or teeth row arrangement between males and females in any pure species or hybrids, so both sexes were analysed together. In the analysis of pure species, *O. lidole* was found to have fewer teeth rows and *O. shiranus* had the greatest count (Table 5.13). Teeth row count was found to differ significantly (P<0.05) between groups, except between *O. shiranus* and *O. karongae* and between *O. karongae* and *O. squamipinnis* (Table 5.14). In the analysis of hybrids, significant differences were found with hybrids (from five farm ponds) and all other groups, although hybrids of just the DWSE and DWST ponds did not significantly differ in teeth row count to those of *O. lidole*.

Table 5.13 Details of teeth rows counts for pure, all hybrid fish and the hybrid fish from two farm ponds and Dwangwa (DWSE and DWST).

Group*	n	Mean	Std.	Range	Median
OSHI	21	4.81	(0.60)	4-6	5
OKAR	24	4.54	(0.78)	3-6	4
OLID	18	3.44	(0.70)	3-5	3
OSQU	21	4.26	(0.45)	4-5	4
HYBRID	47	3.81	(0.74)	3-6	4
DWSE	11	3.91	(0.83)	3-6	4
DWST	21	3.86	(0.79)	3-6	4

* OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; DWSE, Sewage pond Dwangwa; DWST, Storage pond Dwangwa.
Std., Standard deviation.

Table 5.14 Pair-wise comparisons of teeth row count between pure and hybrid fish, and between pure and hybrid fish from two farm ponds separately, tested by Mann-Whitney test (U-statistic and significance).

Group*	OSHI		OKAR		OLID		OSQU	
	U	*P*	U	*P*	U	*P*	U	*P*
OKAR	196.0	n.s						
OLID	37.0	***	68.0	***				
OSQU	126.0	**	221.0	n.s	74.0	***		
HYBRID	155.5	***	286.0	***	299.0	*	327.0	**
DWSE	41.0	**	80.0	*	64.0	n.s	82.5	*
DWST	78.0	***	150.0	*	130.0	n.s	156.5	*

* see Table 5.15. n.s, not significant; **P*<0.05; ***P*<0.005; ****P*<0.001.

Teeth row arrangement varied little between groups; only *O. squamipinnis* had a different median arrangement (coded 1; first row distinct and the following rows overlapping) and no individuals had a teeth arrangement where all rows were indistinct (coded 3) (Table 5.15). The arrangement of *O. squamipinnis* was significantly (*P*<0.005) different from all other groups (Table 5.16). The median teeth row arrangement was the same for all other groups (code 2; all rows distinct).

Table 5.15 Details of teeth row arrangement in pure, all hybrid fish and the hybrid fish from two farm ponds at Dwangwa.

Group*	n	Mean	Std.	Range	Median †
OSHI	21	1.71	(0.78)	1-3	2
OKAR	24	2.04	(0.69)	1-3	2
OLID	18	2.06	(0.42)	1-3	2
OSQU	21	1.04	(0.21)	1-2	1
HYBRID	47	2.11	(0.79)	1-3	2
DWSE	11	2.00	(0.77)	1-3	2
DWST	21	1.95	(0.86)	1-3	2

* OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; DWSE, Sewage pond Dwangwa; DWST, Storage pond Dwangwa.
† Coding for teeth row arrangement: 1, Only the first row is distinct; 2, All rows distinct; 3, All rows indistinct and overlapping. Std., Standard deviation.

Table 5.16 Pair-wise comparisons of teeth row arrangement between pure and hybrid fish, and between pure and hybrid fish from two farm ponds, tested by Mann-Whitney tests (U-statistic & significance).

Group*	OSHI		OKAR		OLID		OSQU	
	U	*P*	U	*P*	U	*P*	U	*P*
OKAR	189.5	n.s						
OLID	132.5	n.s	215.0	n.s				
OSQU	123.5	***	66.5	***	19.5	***		
HYBRID	361.0	n.s	534.0	n.s	398.0	n.s	153.0	***
DWSE	91.5	n.s	128.0	n.s	95.0	n.s	38.5	***
DWST	187.0	n.s	236.0	n.s	174.0	n.s	99.0	***

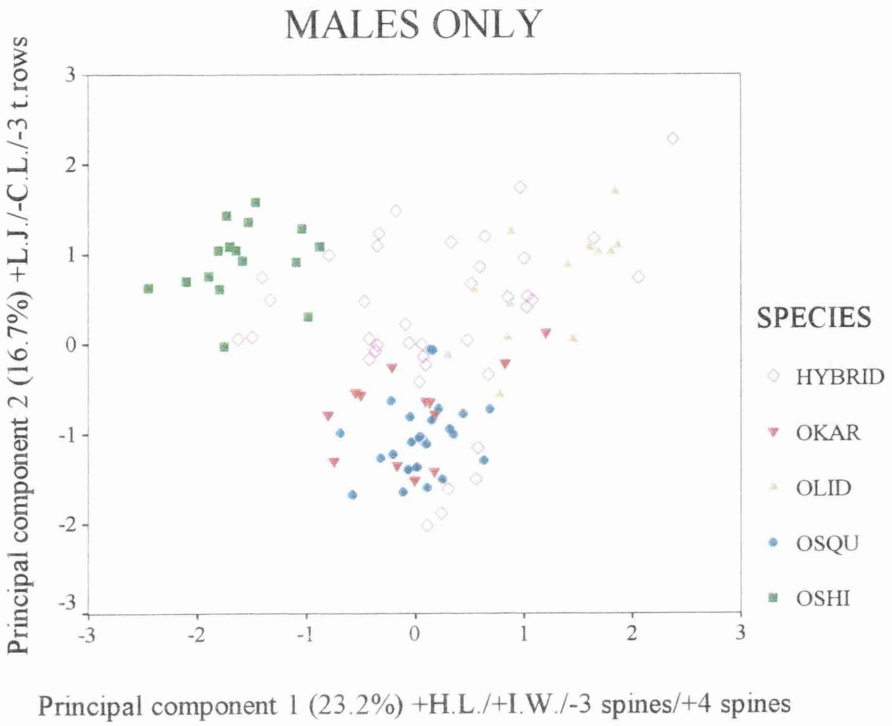
* see Table 5.15. n.s, not significant; ****P*<0.001.

5.3.5 Multivariate analysis of morphological characters

The number of anal fin spines is a meristic character that is quick and easy to count, and is valuable in the discrimination of *O. shiranus* from chambo species; *O. shiranus* have four anal fin spines, whereas, chambo have three anal fin spines. The number of anal fin spines was found to vary between fish in most of the farm ponds, apart from DMOK and MZKH where all fish had three fin spines. Because the eight morphometric characters could not discriminate between all pure species (Figure 5.1 and 5.2), data of teeth row count and arrangement and anal fin spine count were analysed with morphometric data to determine if hybrids and pure species could be successfully distinguished. All these characters are quick and easy to record, can be made on live fish and are not affected by the breeding condition of the fish.

Six PCs were obtained from the PCA of pure species and hybrid groups. The first two PCs explained approximately 40% of the total variance. The heaviest loadings of the first PC were on H.L., I.W. and anal fin spine count. The second PC was also correlated with anal fin spine count, although the heaviest loadings were on L.J. and C.L. The third and fourth PCs (not represented in Figure 5.4) explained approximately 24% of the variance and were most highly correlated with B.D., SN.L. and the variables associated with teeth arrangement (Table IV.2, Appendix IV). In the PCA plot of the first and second PC scores (Figure 5.4), the separation of *O. shiranus* from chambo was better than that obtained with morphometric variables alone (Figure 5.1). The separation of *O. lidole* from the other chambo species was also improved, although there was still much of overlap between *O. karongae* and *O. squamipinnis*. The discrimination between species was slightly improved when males were analysed alone (Figure 5.4). Hybrids did not form a separate cluster from the pure species and were scattered across the whole plot, although there were separate clusters near the *O. shiranus* group, within and around the *O. karongae* and *O. squamipinnis* cluster, and predominantly within and around the *O. lidole* cluster (although these cases are mainly from the DMSH and DWST ponds).

Hybrids formed a separate cluster from the pure species with the more powerful DFA, although a large number of cases fell within the *O. lidole* cluster, particularly when both sexes were analysed together. The discrimination between chambo species was better than that achieved by PCA, although there was still a lot of overlap between *O. karongae* and *O. squamipinnis* in the plot of the first and second DF. As before, discrimination between species was slightly improved when males were analysed alone (Figure 5.5). The first DF explained approximately 50% of the variance and was most highly correlated with anal fin spine count, I.W. and C.L. The variables C.D. and



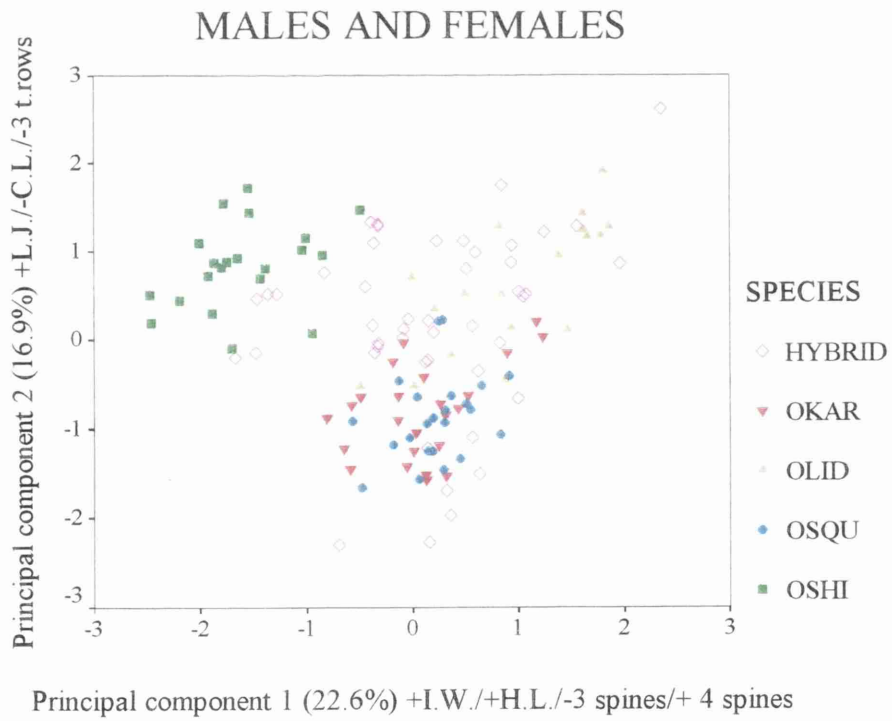
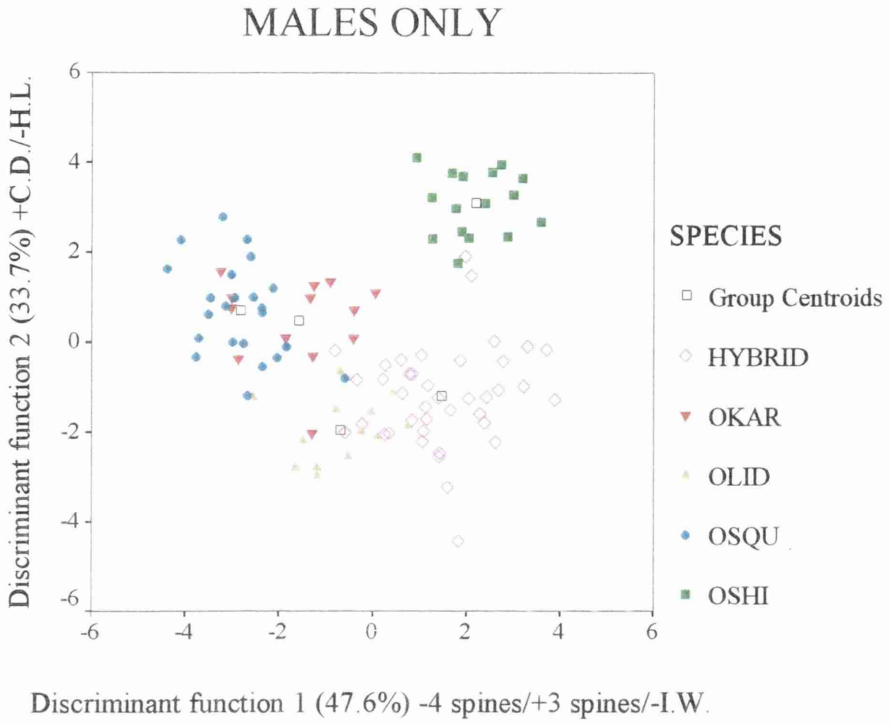


Figure 5.4 Plots of the first and second PC scores from a PCA of morphometric, dentition and anal fin spine count data, of males only, and of male and female pure fish sampled from the wild (OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis;* OSHI, *O. shiranus*) and hybrid fish sampled from farm ponds in Malawi. Variables most correlated with the respective PCs are shown on the axis, with their sign of correlation.



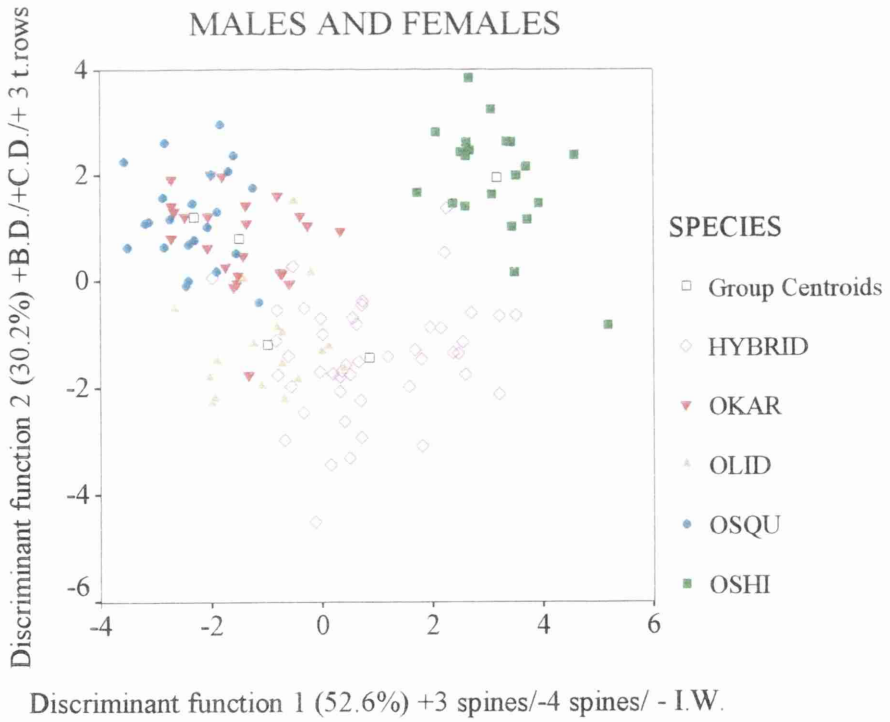


Figure 5.5 Plots of the first and second DF scores from a DFA of morphometric, dentition and anal fin spine count data, of males only, and of male and female pure fish sampled from the wild (OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis;* OSHI, *O. shiranus*) and hybrid fish sampled from farm ponds in Malawi. Variables most correlated with the respective DFs are shown on the axis, with their sign of correlation.

H.L. were highly correlated with the second DF. The within-groups correlations between variables and the two remaining DFs varied when males were tested separately from females. In both tests, the third and fourth DFs were highly correlated with L.J. and teeth row arrangement (Table IV.3, Appendix IV). The classification results of pure species (Table 5.17) were better than that achieved when DFA was performed on morphometric data alone (Table 5.7). The number of cases correctly classified, for all groups, was higher when males were analysed alone than when both sexes were tested together (92.4% compared to 85.2%, respectively). No chambo cases were classified as *O. shiranus*, and all *O. shiranus* cases were correctly classified. Only a few *O. lidole* cases were classified as hybrids. When males were analysed alone, *O. karongae* had the fewest cases correctly classified (84.6%), whereas *O. lidole* had the poorest classification results (72.2%) when both sexes were tested together. The number of hybrid cases correctly classified was improved when males were analysed alone; 90.0%, compared to 80.9% when both sexes were tested together. The remaining hybrid cases were classified to *O. shiranus* and chambo groups.

Table 5.17 Classification results obtained for DFA of morphometric, dentition and anal fin spine data of males only, and of male and female pure fish from the wild and hybrid fish from farm ponds in Malawi. Percentage of cases correctly classified is 92.38% for males only and 85.17% for both sexes together.

			Predicted group membership - percentage of cases									
Actual	no. of cases		OSHI		OKAR		OLID		OSQU		HYBRID	
group*	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀
OSHI	16	21	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OKAR	13	24	0.0	0.0	84.6	83.3	7.7	4.2	7.7	12.5	0.0	0.0
OLID	13	18	0.0	0.0	0.0	11.1	92.3	72.2	0.0	5.6	7.7	11.1
OSQU	23	23	0.0	0.0	4.3	4.3	0.0	0.0	95.7	95.7	0.0	0.0
HYBRID	40	47	5.0	4.3	2.5	2.1	0.0	8.5	2.5	4.3	90.0	80.9

* OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; ♂, males only; ♂ & ♀, males and females.

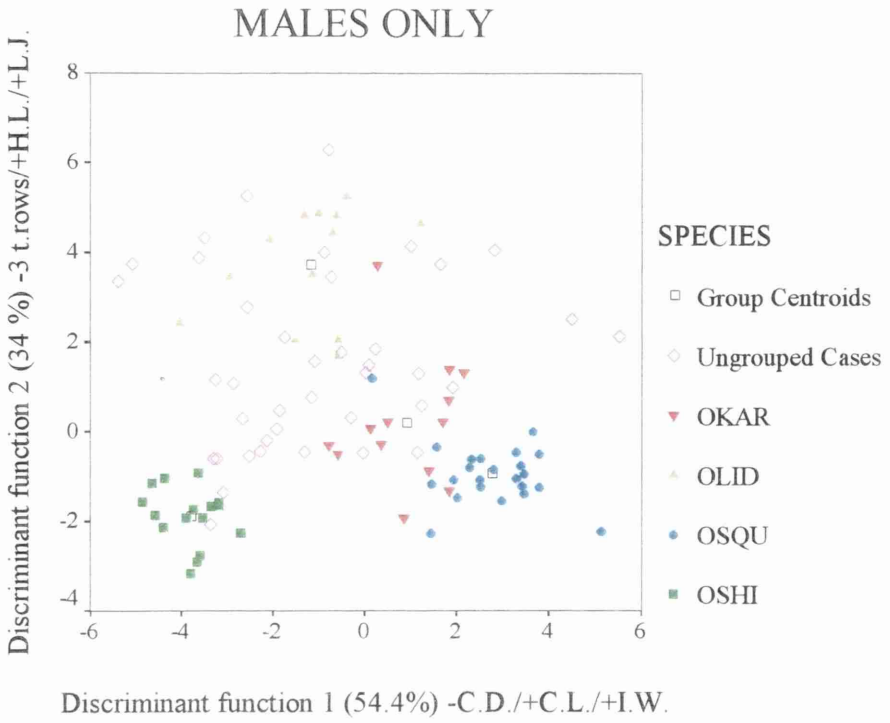
In the DFA of just pure species, there was a higher number of *O. lidole* cases correctly classified, particularly when males were tested alone. Across all groups, 95.4% and 91.9% of cases were correctly classified when males were tested alone and when both sexes were tested, respectively (Table 5.18). Anal fin spine count data could not be included in the analyses, because they were fixed in all groups; four spines in *O. shiranus* and three spines in chambo. Three DFs were obtained; the first and second DF explained over 90% of the variance and were most highly

Table 5.18 Classification results obtained for DFA on size-adjusted measurements and dentition data of males only, and of male and female pure fish, with hybrids from farm ponds as "ungrouped" cases. Percentage of cases correctly classified for pure fish is 95.38% for males only, and 91.86% for males and females. Lower half of the table gives the percentage of hybrids (ungrouped fish) in each farm pond classified to each species group.

Actual	no. of cases		Predicted group membership - percentage of cases OSHI		OKAR		OLID		OSQU	
group*	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀	♂	♂ &♀
OSHI	16	21	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
OKAR	13	24	0.0	0.0	84.6	83.3	7.7	4.2	7.7	12.5
OLID	13	18	0.0	5.6	0.0	5.6	100.0	88.9	0.0	0.0
OSQU	23	23	0.0	0.0	4.3	4.3	0.0	0.0	95.7	95.7
Ungrouped	40	47	30.0	29.8	20.0	19.1	42.5	46.8	7.5	4.3
DMOK	2	2	0.0	0.0	100.0	100.0	0.0	0.0	0.0	0.0
DMSH	9	9	11.1	11.1	22.2	11.1	66.7	77.8	0.0	0.0
DWSE	8	11	75.5	54.5	0.0	0.0	25.0	45.5	0.0	0.0
DWST	18	21	27.8	33.3	5.6	9.5	50.0	47.6	5.6	9.5
MZKH	3	4	0.0	0.0	100.0	100.0	0.0	0.0	0.0	0.0

* OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; DMOK, *O. karongae* pond Domasi; DMSH, *O. shiranus* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storage pond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu; ♂, males only; ♂ & ♀, males and females.

correlated with the variables C.L., C.D., three teeth rows, L.J. and H.L. (Table IV.4, Appendix IV). When the DF scores of pure species were used to predict the classification of hybrid cases, all hybrids from the DMOK and MZKH were classified as *O. karongae*. The majority of hybrid cases from the DMSH and DWST ponds had morphological characters most similar to those of *O. lidole*. The remaining hybrid cases from the DWST pond were classified to all other groups, and those of the DMSH pond were classified as *O. karongae* or *O. shiranus*. Hybrids from the DWSE pond were largely classified as *O. shiranus,* all other hybrids were classified into the *O. lidole* group only (Table 5.18). These findings are reflected in the DFA plot, where hybrid cases are scattered around the *O. lidole, O. shiranus* and *O. karongae* / *O. squamipinnis* group clusters. However, a large number of cases fell outside the main clusters of pure species and some cases were intermediate between the *O. shiranus* and chambo clusters (Figure 5.6). The wide scattering of hybrid cases, particularly those of the DMSH and DWST ponds, demonstrates the morphological distinctness of these from the pure species.



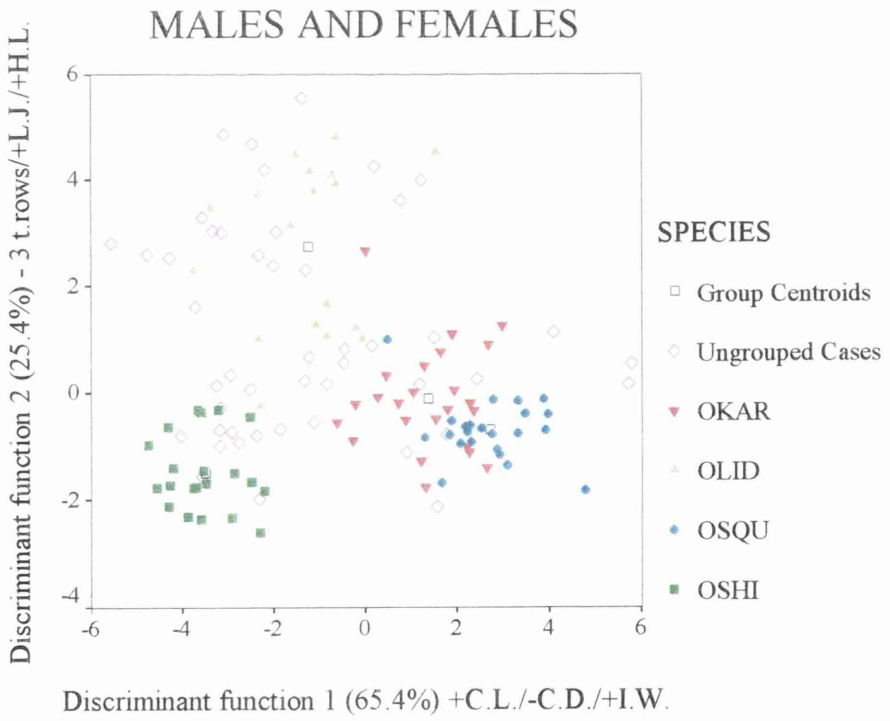


Figure 5.6 Plots of the first and second DF scores from DFA of morphometric, dentition and anal fin spine count data, of males only, and of male and female pure fish sampled from the wild (OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis;* OSHI, *O. shiranus*). This DFA did not include hybrid fish, which were treated as "ungrouped" cases. Variables most correlated with each DFs are shown on the axis, with their sign of correlation .

5.3.6 The discrimination of Domasi farm pond fish from the wild species

Morphometric, dentition and anal fin spine data of the wild species and of fish from the two farm ponds sampled at Domasi (DMOK and DMSH), were submitted to DFA. The incidence of hybridization varied between ponds; hybrids were rare in the DMOK pond and common in the DMSH pond. The DFA was conducted in order to determine whether the morphological discrimination of hybrid fish from wild fish, was due to 'hybrid characters' and not due to farm pond environmental influences alone. Over 80% of the variance was explained by the first DF, which was most highly correlated with anal fin spine count and I.W. (Table IV.5, Appendix IV). In the molecular genetic analyses of the two farm ponds, 20.0% (2/10) and 90.0% (9/10) of the DMOK fish and DMSH fish had been identified as hybrids, respectively (Chapter 3 and 4). Classification results from the DFA predict that all of the farm pond fish are members of their respective groups, that is, fish of both farm ponds could be discriminated from each other and from all wild pure species (Table 5.19). All *O. shiranus* cases were correctly classified, although two chambo cases were classified into the DMOK group.

Table 5.19 Classification results obtained for DFA on morphometric, dentition and anal fin spine count data, of male pure fish from the wild and farm fish from two ponds at Domasi. Percentage of cases correctly classified is 95.29%.

Actual	no. of	Predicted group membership - percentage of cases					
group*	cases	OSHI	OKAR	OLID	OSQU	DMOK	DMSH
OSHI	16	100.0	0.0	0.0	0.0	0.0	0.0
OKAR	13	0.0	84.6	7.7	7.7	0.0	0.0
OLID	13	0.0	0.0	92.3	0.0	7.7	0.0
OSQU	23	0.0	0.0	0.0	95.7	4.3	0.0
DMOK	10	0.0	0.0	0.0	0.0	100.0	0.0
DMSH	10	0.0	0.0	0.0	0.0	0.0	100.0

* OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*; DMOK, *O. karongae* pond Domasi; DMSH, *O. shiranus* pond Domasi;

In the plot of the first and second DF, the DMSH cases formed a cluster that was well separated from all other groups (Figure 5.7). There was a broad overlap of DMOK cases with the *O. lidole* cluster, although values for group centroids (data not shown) of the fourth and fifth DFs differed; these DFs were most highly correlated with the variables for H.L., Arrange 1, SN.L., C.D., and anal fin spine count. If the identity of DMOK fish, based on the genetic analyses is correct, the DFA suggests that farm pond fish can be discriminated from wild populations even if they are pure.

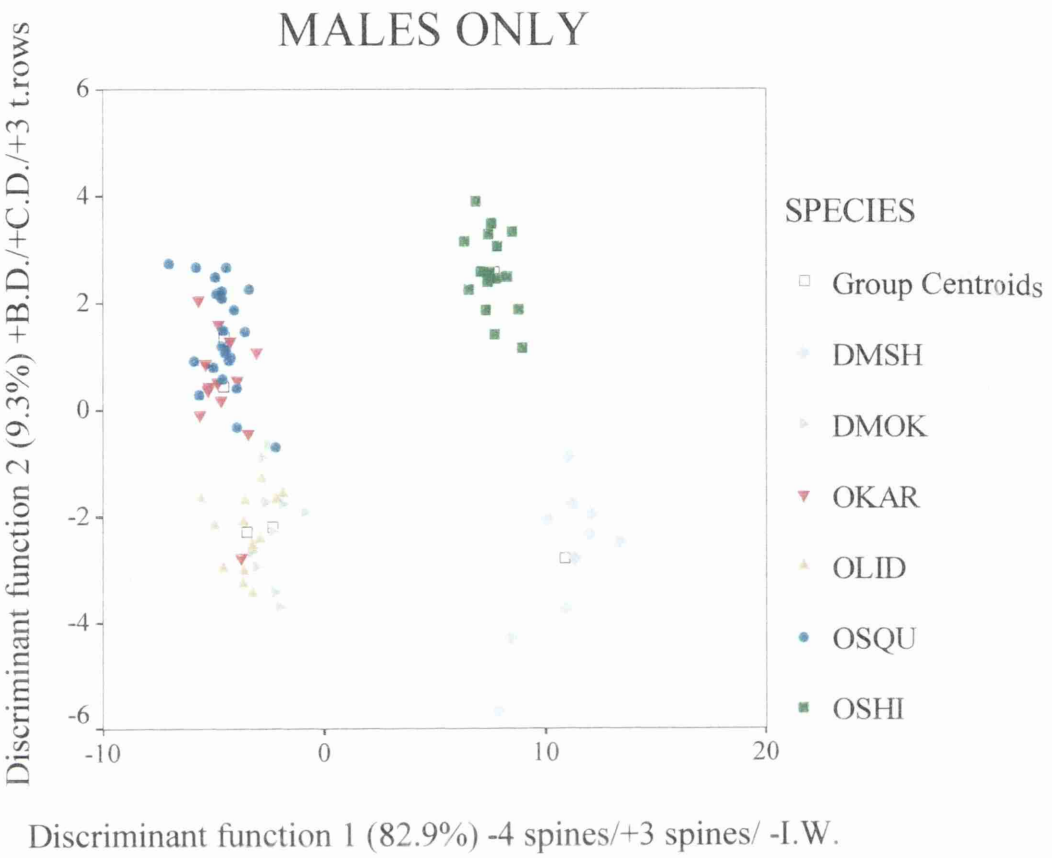


Figure 5.7 Plots of the first and second DF scores from a DFA of morphometric, dentition and anal fin spine count data, of pure fish (males only) sampled from the wild (OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis;* OSHI, *O. shiranus*) and fish from the two ponds at Domasi (DMSH; *O. shiranus* pond and DMOK; *O. karongae* pond). Variables most correlated with the respective DFs are shown on the axis, with their sign of correlation.

5.3.7 Colouration analyses of *Oreochromis* species

The colouration of *Oreochromis* species is heavily dependent on the sex and breeding condition. For example, black body colouration is only seen in ripe males. Species-specific colouration, largely based on that of mature males, was used in the identification of species during sampling (Table 3.1, Chapter 3). Therefore, the colouration data presented in Table IV.6 (Appendix IV) is largely predicted by the characters chosen to identify individuals of each species. For this same reason, just males of *O. squamipinnis* were collected because this species could only be identified reliably by the presence of a male characteristic; the face mask. Body coburation (excluding the head and fins) was often found to be a variety of colours, so the main colours observed were recorded. The colour of the lappets (fleshy tissue) and tips (spinous tissue) of the

dorsal fin margin sometimes differed and were therefore recorded separately. Data for the presence of vertical bars or horizontal stripes, were not used in the analyses because of the large number of missing cases. This was either due to their absence through masking by the body colour of the fish, or to their quick disappearance after death.

On the basis of colouration alone, *O. karongae* males and females were the easiest to distinguish from those of other chambo species by their distinctive coppery body colour. A large number of *O. karongae* also had yellow dorsal fin tips; a characteristic described by Turner & Robinson (1991) as a bright yellow dorsal fin margin seen in females, juveniles and non-breeding fish. A yellow caudal fin margin, not previously described, was observed exclusively in a few *O. karongae* females. Both sexes of *O. shiranus* could be distinguished from chambo species by one or more of the following characters: golden yellow body; orange/red caudal fin margin; orange/red dorsal fin tips. Male *O. shiranus* had the unique character of red dorsal fin lappets, whereas white dorsal fin tips and lappets and white caudal fin margins were only found in males of chambo species. In all species, there were cases that did not possess a caudal fin margin and/or a dorsal fin margin. This could be due to several possible reasons, including sex and sexual immaturity. Furthermore, the caudal fin was often damaged which could have marred the appearance of the margin.

Silver body colouration was observed only in chambo species and olive body colouration in only *O. shiranus* and *O. karongae*. However, the determination of these body colours was very subjective and affected by death, particularly silver colouration which could be marred by grey (therefore, silver was not used in the identification of farm fish). Furthermore, grey and dark grey colouration may be a prerequisite to black in ripe males or the result of a behavioural response in either sex. Few *O. karongae* males were black, which suggests that this species was not in breeding condition at the time of sampling. However, the breeding season of *O. karongae* runs from August to March, as does that of *O. lidole* (Turner & Robinson 1991). Therefore, the lack of black *O. karongae* males may be an artifact of species identification during sampling, rather than breeding condition, caused by the difficulty in discriminating between ripe males of *O. karongae* and *O. lidole*. Copper body colouration was a characteristic largely used in the identification of *O. karongae*, and it is highly likely that black fish could not be discriminated from *O. lidole* with complete confidence and were therefore discarded.

5.3.8 The discrimination of *Oreochromis* species using all variables, and the classification of unknown cases

All variables recorded (morphometric, dentition and colouration data) for both sexes of pure species was submitted to DFA. Anal fin spine count could not be used because counts were fixed in all species. All three discriminant functions obtained explained some of the variance between groups (Wilks' lambda; $P<0.001$), and the highest correlations were found with copper body colour, yellow fin tips, 3 teeth rows, L.J., white fin tips and H.L. The first DF explained 56.5% of the variance and was most highly correlated with copper body colour, olive body colour, red caudal fin margin and C.L. (Table IV.7, Appendix IV). The discrimination of *Oreochromis* species was improved when the analyses included colouration data; only one case was misclassified (Table 5.20). The misclassified *O. karongae* case can be seen to fall into the *O. lidole* region in the scatter

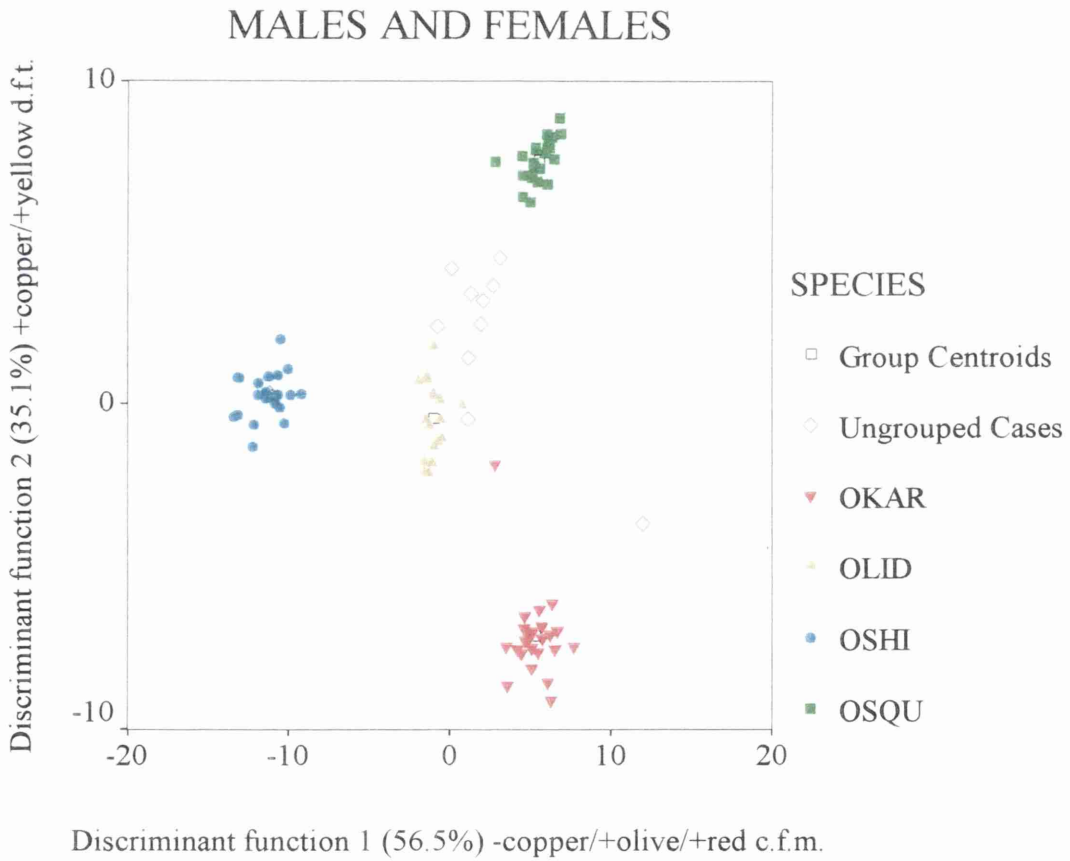


Figure 5.8 Plots of the first and second DF scores from DFA of all morphological variables recorded for wild fish species (both sexes) (OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis;* OSHI, *O. shiranus*) sampled from Lakes Malawi and Malombe. Individuals of unknown identity are also plotted and their functions used to predict their group membership (i.e. species identification). Variables most correlated with the respective DFs are shown on the axis, with their sign of correlation.

plot (Figure 5.8). This individual was a sexually mature male in black breeding colours and, as discussed in section 5.3.7, it is likely that this fish was incorrectly identified during sampling as *O. karongae* rather than *O. lidole*. Of the eleven unidentified fish collected in the wild (see Figure 5.8), five were classified into the groups to which they were suspected to belong. For all cases, the probability of the predicted classification was greater than 99% (Table 5.21).

Table 5.20 Classification results obtained for a DFA on all morphological variables recorded, of male and female fish of four species sampled from Lakes Malawi and Malombe. Percentage of cases correctly classified is 98.84%.

Group*	OSHI n=21	OKAR n=24	OLID n=18	OSQU n=23
OSHI	100.0	0.0	0.0	0.0
OKAR	0.0	95.8	4.2	0.0
OLID	0.0	0.0	100.0	0.0
OSQU	0.0	0.0	0.0	100.0

* OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*.

Table 5.21 Suspected and predicted classification of eleven fish collected from the wild. The predicted classification (and its probability) is based on the DFA of all morphological variables recorded for both sexes of the four species sampled from the wild.

Case no.	sex	Suspected classification	Predicted classification	Classification probability
6	♀	OSQU	OLID	0.999
12	♀	OSQU	OLID	1.000
23	♂	OSHI	OSHI	1.000
85	♂	OKAR	OKAR	1.000
109	♂	OKAR	OLID	1.000
110	♂	OKAR	OLID	1.000
111	♂	OLID	OLID	1.000
112	♂	OKAR	OSQU	1.000
113	♀	OKAR	OSQU	0.999
114	♀	OLID	OLID	1.000
115	♀	OLID	OLID	0.999

♂, male; ♀, female; OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*

5.3.9 The identification of farm pond fish based on colouration

In the study of colouration in farm pond fish it was assumed that each colour was the same as that found in pure wild species, although there is no evidence in this study to support the assumption that the same phenotypic expression is under the same genetic control. The colouration of fish varied between farm ponds, although no farm pond fish were found to have a yellow caudal fin margin or red dorsal fin lappets. The absence of red dorsal fin lappets is surprising since the other *O. shiranus* characteristics, such as orange/red dorsal fin tips and an orange/red caudal fin

margin, were common. The majority of farm fish had dorsal fin lappets that were translucent or indistinct from the rest of the dorsal fin ('no colour') (Table IV.8, Appendix IV). Nine reliable species-specific characters (for *O. shiranus*, chambo or a specific chambo species), as discussed in section 5.3.7, were used in the identification of farm fish. Individuals with the colouration of more than one species were considered as hybrids.

The identity of fish based on colouration alone, and on colouration and fin spine count, are presented in Table IV.9a (for the ponds DMOK, DMSH and DWSE) and in Table IV.9b (for the ponds DWST and MZKH) (Appendix IV). Of the 51 hybrids identified by genetic analyses, only 21 could be considered as hybrids based on colouration alone. If anal fin spine count was also considered, 34 of the fish were classified as hybrids. Of the 19 farm fish classified as chambo species by genetic analyses, four were identified as hybrids with colouration alone. The colouration and identity of fish, based on species-specific colouration alone and when combined with anal fin spine count, will be considered for each farm pond in turn:

1) *O. karongae pond*, Domasi (DMOK): Only male fish were sampled from this pond, of which nearly all had the breeding colouration of mature chambo species; dark grey or black body with white dorsal fin lappets and white dorsal fin tips. All fish had three anal fin spines and a genital tassel; both chambo characteristics. One male also had an orange/red caudal margin, indicative of *O. shiranus*, confirming the genetic identification as a hybrid. All other fish, including one other that was identified as a hybrid with genetic analyses, were identified as chambo. Of these fish, three possessed a face mask, and could therefore be identified as *O. squamipinnis*. No individuals had copper body colouration, indicative of the chambo species *O. karongae*, although this was not possible to ascertain because of the black colouration.

2) *O. shiranus* pond, Domasi (DMSH): All fish had a similar colouration largely indicative of *O. shiranus* (red dorsal fin tips and red caudal fin margin), although they also had white dorsal fin lappets and were therefore all considered as hybrids. The number of anal fin spines varied between fish, but the majority had four spines. No fish had a genital tassel. In genetic analyses, all fish were identified as hybrids, apart from one individual (no. 45) which was identified as *O. shiranus*.

3) Sewage pond, Dwangwa (DWSE): Over 85% of fish were identified as hybrids by genetic analyses, whereas all fish were identified as *O. shiranus* if species-specific colouration was considered alone. However, a large proportion of fish had silver body colouration (only otherwise found in chambo species). When the number of anal fin spine counts is taken into consideration, 20% of fish were identified as hybrids. No fish had a genital tassel.

4) Storage tank pond, Dwangwa (DWST): Nearly 40% of fish, previously identified as hybrids, were found to have a mixture of *O. karongae* and *O. shiranus* colouration characteristics. Most of the remaining fish, which largely had dark grey or black body colouration, were identified as hybrids when anal fin spine count was considered (thereby, over 80% of fish were identified as hybrids), although it was not possible to determine which chambo species was involved. The two fish, identified a chambo by genetic analyses, were also identified as chambo species; both with *O. karongae* characteristics and a genital tassel. Only one other fish sampled from the pond had a genital tassel.

5) *O. karongae* holding pond, Mzuzu (MZKH): All fish had one or more *O. karongae* colouration characteristic and three anal fin spines. Five males also had orange/red dorsal fin tips, which suggests they were hybrids if this characteristic is the same as that seen in *O. shiranus*. Five of the seven males also had a genital tassel. The identification of half of the cases complimented that obtained from genetic analyses. Of the remaining cases, some fish identified as chambo by genetic analyses could be considered as hybrids based on colouration, and the reverse situation was also true.

5.4 DISCUSSION

5.4.1 The morphological discrimination of *O. shiranus* and chambo

The validity of this study, which was primarily to determine if hybrid fish can be identified using morphological characters, is reliant on the correct identification of fish during sampling. Morphological characters were used to determine the unequivocal identity of wild fish during sampling (see Table 3.1, Chapter 3, for species-specific characters). The difficulty in distinguishing between chambo species, particularly between *O. karongae* and *O. lidole*, is illustrated by the eleven chambo fish that could not be identified to the species level during sampling (these fish were not used as representatives of the species during this study). Nevertheless, the correct identification of fish is supported by the results of this study, in which only one case appears to have been incorrectly identified during sampling (see sections 5.3.8 and 5.4.2). Only farm fish that had been decisively identified as hybrids by molecular genetic techniques (Chapter 3 and 4) were used as representatives of the 'hybrid group' during analyses.

The identification and morphological characterisation of hybrids, largely relies on the extent with which parental species can be distinguished. The measurements made were selected to maximize discrimination between the two suspected parental species; *O. shiranus* and chambo.

These species can be distinguished by anal fin spine count alone, although the identification of hybrids would not be possible because individuals have only one of the characters; either three or four anal fin spines. The successful discrimination of *O. shiranus* and chambo was achieved, at a level when 95% of cases were correctly classified, when eight size-adjusted morphometric characters were analysed. The necessity of all eight morphometric characters for the discrimination of *O. shiranus* and chambo was not determined, although univariate analyses suggests that maximum body width (M.W.) was not valuable. In fact, *O. shiranus* was characterised by three variables; those associated with caudal peduncle shape (C.D. and C.L.) and interorbital width (I.W.). *O. shiranus* has a deeper and shorter caudal peduncle, and a shorter interorbital width, than that of chambo. All *O. shiranus* individuals were correctly classified when teeth row count and teeth row arrangement were included in the analyses. Teeth row count was higher in *O. shiranus*. Teeth arrangement did not differ significantly between groups, and was therefore unlikely to be of importance in the discrimination of *O. shiranus* and chambo.

5.4.2 The morphological discrimination of chambo species

Chambo species could not be discriminated successfully using the eight morphometric characters alone. However, these characters were originally selected to maximise discrimination between *O. shiranus* and chambo. In the study of Turner *et al.* (1989b), the discrimination of chambo species, based on material held at the BMNH, was found to be dependent on the inter-relationship of many measurements; 18 measurements (expressed as ratios with standard length) were required to discriminate between all chambo species at the 95% classification level. To discriminate between pairs of individual species, 8 to 12 variables were required. Many of the most useful measures were not standard taxonomic measures (e.g. depth at jaw angle, depth at posterior of eye, depth at preopercular and opercular measures). In the former study, four species were thought to comprise the *Oreochromis (Nyasalapia)* flock of Lake Malawi. In a later study, it was concluded that *O. saka* was a junior synonym of *O. karongae* (Turner & Robinson 1991).

Here, the variables associated with head shape (head length, lower jaw length and snout length) were most valuable in discriminating between chambo species. Discrimination between species was improved if males were analysed alone, particularly for *O. lidole* where a large number of measurements significantly differed between sexes. Coefficients of variation (CV) in measurements were generally greater in all species when both sexes were analysed together. Sexual dimorphism has been found in the morphometric studies of other species of tilapia (Brzeski &

Doyle 1988; Velasco *et al.* 1993). In this study, it was not possible to analyse females alone due to the small number of samples. The reduction in the amount of discrimination between species observed when both sexes were tested together suggests that variation between females is less than that observed between males of each species. Alternatively, shape variation between females of each species may be different to that observed between males. It would be valuable to test the morphological variation between females in a future study. Nevertheless, the ability to discriminate between chambo and *O. shiranus,* when both sexes are analysed together, is valuable in studies where it is not possible to sex all individuals.

The discrimination between chambo species was improved when dentition data were included in the analyses. However, the 95% classification level was only achieved for *O. squamipinnis* (when males were tested alone and with females) and for *O. lidole* (when males were tested alone). Nevertheless, the discrimination of chambo species was not possible at all with allozyme and RAPD analyses (Chapters 3 and 4). *O. lidole* was characterised by a reduced dentition (3-5 rows), in comparison to *O. squamipinnis* (3-4 rows) and *O. karongae* (3-6 rows). No significant differences were found in teeth row count between *O. squamipinnis* and *O. karongae,* although the arrangement of *O. squamipinnis* teeth rows was significantly different from all other species. Turner & Robinson (1991) reported considerable habitat-related geographic variation in the teeth row count of *O. karongae*: 4-6 rows in fish sampled from the northern population at Korongae; 4-14 rows in the Cape Maclear population; and 3-9 rows in the Lake Malombe population. Populations of the SE Arm and SW Arm were intermediate between the Cape Maclear and Malombe populations. In this study, where fish were sampled from the SE Arm of Lake Malawi, the maximum number of teeth rows was relatively low, although the number of fish studied was comparatively small. Turner & Robinson (1991) also found geographic variation, in the sites mentioned above, in the pharyngeal bone and body shape of *O. karongae. O. lidole* had a fairly constant morphology across the lake, whereas the pharyngeal bone and body shape of *O. squamipinnis* varied between northern and southern populations. Therefore, the samples in this study, particularly those of *O. karongae* and *O. squamipinnis*, are unlikely to be representative of species across the whole lake. Significantly higher levels of morphological variability (based on the CV of morphometric variables) were found in males of *O. karongae* compared to those of *O. lidole* and *O. squamipinnis.* However, *O. karongae* was sampled from more locations than the other two species.

When colouration data were included in the analyses of *Oreochromis* species (of both

sexes), only one case was misclassified; as *O. lidole* rather than *O. karongae*. It is likely that this fish was incorrectly identified during sampling, due to the difficulty in distinguishing between sexually mature *O. karongae* and *O. lidole* males, or due to human error. High classification results allowed the confident identification of the unknown cases to be made, whereas molecular genetic analyses only allowed unknown cases to be identified as *O. shiranus* or chambo.

5.4.3 The identification and characterisation of hybrids based on morphology

The reliable identification of all hybrid cases was not possible using the small number of morphological characters, despite the ability to distinguish between *O. shiranus* and chambo. Up to 90% of cases were correctly assigned to the hybrid group when males were tested alone, but only 80.9% of cases were correctly classified when both sexes were analysed together. One explanation for these findings is the extensive backcrossing of farm pond individuals. Introgressed populations often appear morphologically identical to one of the parental species (Busack & Gall 1981; Campton 1987; Lamb & Avise 1987). In this study, a large number of hybrid cases were found to cluster around the groups of pure species, particularly in the PCA plot, although hybrids did form a separate cluster from the pure species in the DFA plot. Neff & Smith (1979) criticized the use of DFA because it requires *a priori* identification of the hybrid and is very prone to produce the groups one initially provides. However, the poor discrimination of hybrids seen in the PCA plots may be because less than 40% of the variance was explained by the first two PCs that were used to produce the plot.

Some hybrid cases were positioned between the *O. lidole* and the *O. karongae / O. squamipinnis* groups, which may suggest that they had an intermediate morphology and could be hybrids between these species. Colouration data did not support this hypothesis because no individuals were found to have the characteristics of two chambo species. However, there were few colouration characters that could be used reliably to distinguish between chambo species. A few hybrid cases were found between *O. shiranus* and chambo groups, when hybrid cases were 'ungrouped'. Overall, the morphology of hybrid cases was not intermediate between any species which supports the genetic evidence that hybrids were not of the first generation (see Chapters 3 and 4). However, the intermediacy of F_1 hybrids cannot be presumed (Campton 1987). Studies of artificially produced salmon/trout hybrids have shown that there are hybrid individuals where characteristics of one parent appear to be dominant or partially dominant over those of the other parent (Jones 1947; Nyman 1970). Experimentally produced F_1 hybrids between the Lake Malawi

haplochromine cichlids *Pseudotropheus zebra* and *Labeotropheus fuelleborni*, although morphologically distinct, more closely resemble *P. zebra* than *L. fuelleborni* (McElroy & Kornfield 1993). Furthermore, artificial hybrids between two subspecies of *Esox americanus* scored as intermediate in discriminant analysis, although in some characters the hybrids more closely resembled the male parents (Reist & Crossman 1987). In this study, the absence of red dorsal fin lappets in hybrids may be due to the dominance of other 'non-*O. shiranus*' colours. However, the occurrence of intermediacy in hybrids between the *Oreochromis* species of Lake Malawi and/or the dominance of parental characters can only be determined if F_1 hybrids are experimentally produced.

The morphological distinctness of many hybrid individuals, observed in the clustering of the hybrid group and the positioning of hybrid cases outside the clusters of pure species when hybrids were ungrouped, may be a result of the unique genetic recombination of parental genomes in hybrids. The disruption of coadapted gene complexes in hybrids may reduce developmental stability in comparison to their parental species (Leary *et al.* 1985). Leary *et al.* (1983) suggested that high counts in the meristic characters of hybrids between the brook trout (*Salvelinus fontinalis*) and bull trout (*S. confluentus*), where counts were higher than either parental species or similar to the parental species with the higher count, was due to a reduced developmental rate caused by genetic incompatibility between the parental genomes. In this study, hybrids did not have counts higher than, or as high as the parental species, although only two meristic counts were made (teeth rows and anal fin spines). Neff & Smith (1979) attributed the larger eyes of hybrid individuals, in comparison to similarly-sized individuals of the parental species *Notropis spilopterus* and *N. whipplei*, to the combination and interaction of dissimilar developmental rates. This can result in high hybrid variability relative to the parental forms. In this study, some hybrid individuals of the DWST and DWSE had noticeably larger eyes than the parental species (other explanations for this character are discussed later). Furthermore, many of these fish also had deformed jaws. In a multivariate study of the oral morphology, of experimentally produced F_1 hybrids between *P. zebra* and *L. fuelleborni*, a number of hybrids were found to display unique patterns (McElroy & Kornfield 1993). The creation of novel genotypes can contribute to the increase in morphological variability caused by the occurrence of more than one species in an intergrade population (Wilde & Echelle 1997). In this study, the CVs of all hybrids combined, and hybrids from just the DWSE and DWST ponds, were found to be significantly higher than those of the pure species. If it is assumed that each quantitative trait indicates variation at several loci (Berry & Jakobson 1975;

Hartl *et al.* 1994), then morphological variation can be used to estimate genetic variation (Falconer & Mackay 1996). Echelle & Conner (1989) found that geographic variation in belly scaling, roughly paralleled that observed in allozymes of intergrade pupfish (*Cyprinodon*) populations. Furthermore, the morphological intermediacy of some hybrids provides evidence for the genetic control of morphological characters (Reist & Crossman 1987), although there is also extensive evidence for the environmental modification of characters (discussed later). In this study, allozyme analysis (Chapter 3) indicated that genetic variability was higher in Malawi farm pond populations, although this analysis was performed on all farm pond individuals and not just those identified as hybrids.

The discrimination of DMOK farm fish, where the incidence of hybridization was low, suggests that the unique morphologies of hybrids may also be caused, to some extent, by the environmental effects of aquaculture. Alternatively, the discrimination of DMOK fish may also be because the morphological variability of wild populations, seen as geographic variation (Turner & Robinson 1991), was not sufficiently represented in the samples of wild fish. This may be particularly true for the farm ponds at Mzuzu, which is to the north west of Malawi, because samples of wild fish were taken from the southern region of Lake Malawi only. However, it is not known whether the variation in trophic structures shown by *O. karongae* and *O. squamipinnis* is genotypic, purely a phenotypic response to the dominant diet experienced in different habitats, or results from a combination of factors (Turner & Robinson 1991). There is extensive evidence for the environmental modification of morphological characters in fish, through factors such as diet (Robinson & Wilson 1995) and temperature (Beacham 1990). Shape differences have also been found between hatchery reared and wild salmon, *Salmo salar*, of common genetic stocks (Fleming *et al.* 1994). The introduction of *O. mossambicus* into foreign countries has led to morphological changes in this species because the fish were brought in contact with different environmental factors (Van der Bank & Ferreria 1987). The size of water bodies has been found to induce size differences in *O. mossambicus* (Van der Bank & Ferreira 1987), and the smaller maximum size of fish from smaller water bodies is thought to result from stunting due to over population from uncontrolled reproduction (Chen & Prowse 1964; Lovshin & De Silva 1975). Noakes & Balon (1982) suggested that stunting in tilapias is a response to the introduction into a new habitat (i.e. farm pond) where there is a shift towards a more altricial life style, characterised by accelerated sexual maturation and a reduced interval of somatic growth. In this study, the uncontrolled reproduction of fish and over population of farm ponds was evident, particularly in the DWSE and

DWST populations. Fish from these ponds typically had a large head and tapering body; phenotypic characteristics typical for stunted populations. Stunting may have resulted in the large eyes of these fish; a study of rainbow trout, *Oncorhynchus mykiss*, demonstrated that individuals with reduced somatic growth have larger eyes (Pankhurst & Montgomery 1994).

The shape of stunted fish (large head and tapering body) is similar to the characteristic shape of *O. lidole*. This may explain why some of the hybrid farm pond fish, particularly those from the DMSH and DWSE ponds, were classified as *O. lidole*. Furthermore, a small number of *O. lidole* cases were classified into the hybrid group. The predicted identity of these farm fish, based on the discriminant function scores of pure species, is questioned because they do not compliment the identities based on colouration. For example, the majority of fish from the DMSH pond largely had the colouration, and number of anal fin spines, characteristic of *O. shiranus*, although very few cases were classified as *O. shiranus* in the analyses of morphological characteristics. The only indication of a hybrid identity was based on the presence of white dorsal fin lappets. Furthermore, genetic analysis indicated that these fish were the progeny from hybrid backcrosses with *O. shiranus*. Approximately half of the hybrid fish from the DWST pond had morphologies most similar to *O. lidole*, approximately a third were classified as *O. shiranus* and very few were classified as *O. karongae*. However, colouration characteristics suggest a lot of these fish were hybrids between *O. karongae* and *O. shiranus*. The inability of morphological characters to distinguish between *O. karongae* and *O. squamipinnis*, may explain why the three fish from the DMOK pond found to have a face mask (indicative of *O. squamipinnis*) were classified as *O. karongae* on the basis of morphological characters.

Less than half of the hybrid fish, identified with genetic analyses, could be identified as hybrids using colouration characteristics. Whereas, approximately 67% of hybrids were identified using colouration and anal fin spine count and a further four individuals, identified as 'pure' fish with genetic analyses, were identified as hybrids. However, colouration characteristics are heavily dependent on the sex and breeding condition of the fish, and the identification of hybrids was often based on only a few colours. Furthermore, there is no genetic evidence in this study to support the assumption that the same phenotypic expression is under the same genetic control. The body colouration of cichlids can change in response to environmental changes and these changes reflect different phases of behaviour (Rothbard 1979; Neil 1964; Lanzing & Bower 1974). Furthermore the body colouration has been found to be influenced by the type of background colour (Lanzing & Bower 1974). The general extent of environmental modification of colouration in cichlids

appears limited, although diets can affect the intensity of colouration, particularly reds (Kornfield 1991). Nevertheless, species-specific colouration characteristics, combined with anal fin spine count, may be valuable in indicating the presence of hybrids before a more extensive genetic study is performed.

5.5 SUMMARY

The findings of this study can be summarised as follows:

1) *O. shiranus* and chambo can be discriminated using a small number of morphometric characters.

2) The discrimination of chambo species, using morphometric characters, was improved when males were analysed separately from females. However, the reliable discrimination of chambo species requires the analyses of morphometric and colouration characters. Colouration is heavily dependent on sex and breeding condition. Unlike the use of molecular genetic analysis, it was possible to predict the identity of chambo individuals that were unidentified during sampling.

3) The morphological characters used in this study, cannot reliably distinguish all hybrid individuals. This was most likely due to extensive backcrossing, which has resulted in hybrids resembling one of the parental species. Very few hybrids had a morphology intermediate to that of the parental species. The study of experimentally produced F_1 hybrids would be valuable in determining the characteristics of hybrids between the *Oreochromis* species of Lake Malawi.

4) Some hybrids were found to have a morphology unlike either parental species. This morphological uniqueness may be due to the interaction of parental genomes (which may lead to developmental instability), the environmental effects of aquaculture, or because the wild phenotype was not sufficiently defined (due to insufficient sampling).

5) Species-specific colouration characteristics are generally poor at identifying introgressed hybrids, although when combined with anal fin spine count may be valuable in indicating the presence of hybrids prior to extensive genetic studies.

CHAPTER 6
GENERAL DISCUSSION

6.1 INTRODUCTION

The unintentional hybridization of *Oreochromis* species in aquaculture, and in open waters following introductions, is not an uncommon phenomenon. The effects of hybridization have frequently been detrimental, often contributing to the elimination of a native species following an introduction (Moreau 1983; Ogutu-Ohwayo & Hecky 1991). This study has largely been concerned with estimating the incidence of hybridization in farm ponds between the *Oreochromis* species native to Lake Malawi (*O. shiranus* and the three *Oreochromis (Nyasalapia)* species). The aim of this final chapter is to review the evidence for the presence of hybrids in Malawian farm ponds, to evaluate the efficiency of the three techniques employed in identifying species and hybrids, and to assess the implications of hybridization on farm pond populations and on wild populations. This study is of particular poignancy because of the uniqueness of the *Oreochromis (Nyasalapia)* species flock (chambo), and the possible consequences on the many other endemic fish species of Lake Malawi. Recommendations for the management of *Oreochromis* stocks, aimed at preventing hybridization and maintaining genetic diversity, will be considered in relation to the conflicting environmental and social issues.

The ease with which *Oreochromis* species hybridize, sometimes producing offspring that are as fertile and viable as the parental species, complicates the biological species concept. It also brings into question how speciation has occurred and how reproductive isolation is maintained in this group of fish. Geographic separation plays a predominant role in the speciation and isolation of *Oreochromis* species, although allopatric species appear not to have diverged sufficiently in mate recognition systems to prevent interspecific matings. Nevertheless, it has been shown that some allopatric species, at least, have the ability to mate assortatively (this study, Chapter 2; Falter & Charlier 1989), but retain the ability to mate with non-conspecifics when they have no other option (either because conspecifics are not available or receptive). Mechanisms that maintain reproductive isolation between the sympatric *Oreochromis (Nyasalapia)* of Lake Malawi are poorly understood. In the final section of this chapter, the evolutionary origins (i.e. process of speciation) and possible mechanisms that maintain reproductive isolation between these sympatric species will be considered. Particular attention will be given to the role of sexual selection, in reference to recent publications on the rock-dwelling haplochromine species (mbuna) of Lake

Malawi. Mbuna, and the other mouthbrooding cichlids that occupy the other Great Lakes of Africa, are of particular scientific interest due to their diversity and speed of speciation. Reasons as to why the *Oreochromis* species of Lake Malawi are not as diverse as the haplochromine cichlids will be discussed.

6.2 HYBRIDIZATION IN TILAPIA

6.2.1 Introductions and hybridization of tilapia

Due to their adaptability, ease of culture, short generation time, capacity to breed year-round and fast growth rate (Fryer & Iles 1972; Pullin & Capili 1990), tilapia are an increasingly important component of aquaculture in many tropical and subtropical countries. Recent publications report that tilapia (wild types and genetically modified individuals) have been introduced to about 90 countries (Pullin *et al.* 1997). Introductions have been largely intended to boost fisheries production, though in some cases other reasons are prevalent such as mosquito control, weed control, and to fill an ecological niche. Releases are, however, often accidental (Casal & Bartley 1977; Carvalho & Hauser 1995; Pullin *et al.* 1997). Despite many reports of adverse effects to endemic tilapiines and other species following introductions or translocations (reviewed in Pullin *et al.* 1997), very few appraisals of environmental risks have been published (exceptions to this include Eknath *et al.* 1993, INGA 1996). The ease with which *Oreochromis* species hybridize following introductions has also often been overlooked. Introductions to regions where they are not endemic have frequently resulted in hybridization with other introduced (Welcomme 1984, 1988; Arthington 1991; Daget & Moreau 1981; Amarasinghe & De Silva 1996) or native tilapiines (Welcomme 1966; Elder *et al.* 1971; de Moor & Bruton 1988; Ogutu-Ohwayo & Hecky 1991). Most cases where hybridization of *Oreochromis* species has occurred in the confinement of aquaculture breeding ponds, have involved formerly allopatric species (Taniguchi *et al.* 1985; Macaranas *et al.* 1986; De Silva & Ranasinghe 1989; Gregg *et al.* 1998).

6.2.2 Evidence of hybridization in the farm ponds of Malawi

The three endemic *Oreochromis (Nyasalapia)* species (chambo) once formed the bulk of the commercial catch from Lake Malawi, but the increasing demands from an expanding human population and the introduction of new fishing methods have resulted in the loss of three of the four principal chambo fishing grounds (Pitcher & Hart 1995; Turner 1995). The remaining viable chambo fishery in the SE Arm of Lake Malawi appears to be fully exploited. The increasing use

of fine mesh nets, in which large number of immature fish are caught, is also likely to be detrimental to the tilapiine stock (Turner 1995). Therefore, the aquaculture of tilapiines may become an increasingly necessary option. The aquaculture of *O. shiranus* (of little commercial importance to fisheries) and *O. karongae,* is expanding rapidly (Brummett 1995). Local communities are also being encouraged to culture the non-endemic species *O. mossambicus* in ponds and reservoirs (Chikafumbwa *et al.* 1997), although the Malawi government has banned the introduction of other exotic species. A principal objective of this study was to confirm the anecdotal evidence of A. Brooks and G. F. Turner (*pers. comm.*) that interspecific hybrids of *O. shiranus* and *O. karongae* are present in Malawian farm ponds. There is no evidence to suggest that hybridization has occurred between *O. shiranus* and chambo, or between chambo species, in the wild (Turner *et al.* 1991b).

6.2.2.1 Discrimination of the Oreochromis *species of Lake Malawi and the identification of hybrids*

Allozyme, RAPD and morphological analyses were employed to identify the putative interspecific hybrids. Species-specific markers that distinguished between *O. shiranus* and chambo were found with both molecular genetic techniques, and the discrimination of *O. shiranus* and chambo was possible using only eight morphometric variables. However, diagnostic species-specific markers for the three species of chambo were not detected with either allozyme or RAPD analysis. RAPD analysis revealed no bands that were unique to individual chambo species, although allozyme analysis identified several unique alleles. Five alleles were unique to *O. squamipinnis*, and three alleles were observed only in *O. karongae* and *O. lidole,* although all were at low frequencies. The reliability of these alleles as species-specific markers is questionable due to the small sample sizes and conflicting results from the study of Sodsuk *et al.* (1995). Nevertheless, no farm pond fish were found to have genotypes with the 'diagnostic' alleles of more than one chambo species. The discrimination of chambo species was achieved using the multivariate analyses of morphological variables (based on dentition, morphometrics and colouration), in which over 98% of chambo fish were correctly classified. These high classification results allowed confident predictions to be made on the identity of unidentified cases, whereas molecular genetic analysis only allowed cases to be classified as *O. shiranus* or chambo. Principal component analysis of morphometric and dentition data revealed that some farm pond fish had a morphology intermediate to that of the *O. lidole* and *O. karongae* / *O. squamipinnis* groups. Such results suggest that these fish could be hybrids between chambo species, although no individuals

had the colouration characteristics of more than one chambo species. Therefore, only hybrids between *O. shiranus* and chambo were identified.

The absence of fixed genetic differences between chambo species, both in allozyme (supported by Sodsuk *et al.* 1995) and RAPD analysis, and the ability to distinguish between these species with morphological characters (supported by Turner *et al.* 1989), suggest that morphological change in these species has been accompanied by very little genetic differentiation. Indeed, the low F_{ST} value obtained by allozyme analysis of chambo species, is more typical of levels found within a species than between species. Diagnostic markers for chambo species would allow the complete identification of hybrids between *O. shiranus* and chambo, and the identification of hybrids between chambo species. Relatively few RAPD primers were examined in this study, though there are many others available. Alternatively, a more sensitive molecular genetic technique may reveal fixed differences between the chambo species, but only if these species have diverged sufficiently. RFLP analysis of mtDNA has been used to discriminate among subspecies of *O. niloticus* (Seyoum & Kornfield 1992a), and a preliminary study has revealed fragments that were diagnostic for *O. lidole* (Turner & Robinson 1991). However, since mtDNA is inherited maternally, hybrids can be identified only when the analysis is combined with another molecular technique. In such studies, mtDNA analysis has been valuable in detecting the level of introgression (e.g. Dowling & Brown 1989), and the dynamics of interspecific mate choice (e.g. Avise & Saunders 1984; Konkle & Philipp 1992). Nevertheless, mtDNA sequence divergence is very low between the morphologically diverse haplochromine species of Lake Malawi (Moran *et al.* 1994), and may not reveal fixed differences between all chambo species.

The high mutation rate and associated high levels of polymorphisms of microsatellite loci make them of particular value in studies where genetic differentiation may be limited (Wright & Bentzen 1994; O'Connell & Wright 1997), such as between the recently diverged haplochromines where insufficient time has passed for genetic drift to have much affect on variation in allele frequencies (Kornfield & Parker 1997; Sültmann & Mayer 1997). Microsatellite loci have proven be very useful for resolving population structure in mbuna and in demonstrating assortative mating in populations not formally recognised as species (van Oppen *et al.* 1997a, b). Microsatellite variation has also been used to demonstrate multiple paternity in the mouthbroods of haplochromine species (Kellogg *et al.* 1995; Parker & Kornfield 1996). Furthermore, the preliminary analysis of two microsatellite loci in three congeneric pairs of mbuna species strongly suggests that these markers can provide phylogenetic information relevant to these recently

diverged taxa (Kornfield & Parker 1997). Microsatellites have recently been used for the phylogenetic analysis of *Oreochromis* species (including *O. karongae*) and the discrimination of *O. shiranus* subspecies (A. Ambali *pers. comm.*). The isolation of microsatellite markers from *O. niloticus* has also been described (Lee & Kocher 1996). Highly polymorphic microsatellite loci may have great potential for revealing variation between closely related, recently diverged species like the *Oreochromis (Nyasalapia)* of Lake Malawi.

6.2.2.2 The incidence of hybridization between O. shiranus *and chambo in farm ponds, Malawi*

Hybrids of *O. shiranus* and chambo were detected in all of the seven farm ponds sampled from three sites in Malawi (at Domasi, Dwangwa and Mzuzu). It was not possible to determine which species of chambo were hybridizing with *O. shiranus* although, based on morphological characteristics (particularly colouration), *O. karongae* was present in most ponds. Fish with colouration indicative of *O. squamipinnis* were found in one pond. The incidence of hybridization varied between sites, although no F_1 hybrids were detected in any samples and introgressive hybridization was common place. Discrepancies in the identification of several farm fish (40%) between RAPD and allozyme analysis, emphasize the merits of using more than one type of analysis in situations where extensive backcrossing has occurred. RAPD analysis (using nine markers generated from four primers) was not as effective at detecting hybrids as allozyme analysis (using four diagnostic loci; 13 loci examined in total). The analysis of morphological variables was not as effective as either of the molecular techniques, although it was valuable for the preliminary identification of hybrids. Furthermore, a few individuals identified as pure fish with molecular analysis were identified as hybrids based on diagnostic morphological characteristics. The difficulty in identifying hybrids with morphological variables, and the general lack of hybrid individuals with morphologies intermediate to that of the parental species (presuming that F_1 hybrids have an intermediate morphology), supports the molecular genetic evidence of extensive backcrossing. Since later generation backcross hybrids may have genotypes (and phenotypes) resembling those of the parental species, the number of hybrids detected can only be considered as minimum estimates for the incidence of hybridization.

The incidence of hybridization was largely related to whether any preference for stocking of one species (either *O. shiranus* or chambo) had occurred. For example, the sewage pond at the Dwangwa sugar cane plantation had been indiscriminately stocked with fish that entered via an irrigation pipe and over 90% of fish sampled were hybrids. The *O. karongae* holding pond at the

Mzuzu aquacultural centre had intentionally been stocked with *O. karongae* and less than 15% of fish sampled were identified as hybrids (incidence of hybridization based on combined results from allozyme and RAPD analyses). Nevertheless, the greatest incidence of hybridization was detected in the storage pond at Dwangwa, where *O. karongae* had supposedly been chosen for stocking. However, these fish were taken largely from the sewage pond and were unlikely to be pure *O. karongae*; illustrating the dangers of using stocks of unknown genetic identity. In general, the post-F_1 hybrid fish had genotypes that most greatly resembled the species chosen for stocking. For example, hybrid fish from the *O. karongae* holding pond at Mzuzu, were progeny of backcrosses between hybrids and *O. karongae*.

Both farm ponds at Dwangwa were particularly densely populated and breeding was extensive; conditions that would encourage hybridization. The high incidence of back-crossed hybrid individuals strongly suggests that introgression has occurred. All farm ponds had conditions that would prevent the reproductive isolation of species and thereby encourage hybridization. Such conditions include poor water clarity (reducing the ability to identify conspecific mates), lack of habitat separation, limited nesting space, lack of temporal isolation and a scarcity of conspecific mates (for the species that was not chosen for stocking). A scarcity of conspecific mates, in which case *Oreochromis* females appear to mate with the next best option (supported by mate choice experiments in this study), is probably a major factor contributing to hybridization following introductions. Recent studies by Seehausen *et al.* (1997) have highlighted the importance of water clarity in maintaining reproductive isolation through mate choice based on species-specific colouration. In mate choice experiments conducted under monochromatic light, which mask colour differences, females no longer preferred conspecific mates (Seehausen & van Alphen 1998). Furthermore, increased turbidity in Lake Victoria due to human activities, is causing a breakdown of reproductive barriers between cichlid species (Seehausen *et al.* 1997).

Apart from the presence of hybrids, the farm pond populations studied had several features that were characteristic of hybrid populations or indicative of the presence of more than one subpopulation. Allozyme analysis allowed the most detailed examination of population structure, although supporting evidence was provided with RAPD and morphological analyses. Deviations from Hardy-Weinberg equilibrium (HWE), due to a deficiency of heterozygotes, were observed (in combinations over all loci) at three of the five farm pond populations analysed. Apart from the presence of hybrids, deviations from HWE may also be due to due to genetic drift or the Wahlund effect. However, such deviations were also observed in the wild populations of pure species. These

results may have been due to several reasons, including subpopulation of wild species, insufficient and unrepresentative sampling of wild populations, and/or statistical artifacts associated with small sample sizes. Significant linkage disequilibrium, due to the mixing and interbreeding of two or more previously isolated populations, was observed only in the storage tank pond sampled at Dwangwa. A lack of significant linkage disequilibrium in the other farm ponds studied was surprising, although this may be due to extensive backcrossing and the subsequential introgression.

The farm pond populations generally exhibited higher levels of genetic variability and heterozygosity, than the wild populations. Apart from the presence of more than one population or species in farm ponds, increased genetic variability may also be due to the creation of new genotypes through the unique genetic recombination of parental genomes in hybrids. Variability in cultured tilapia populations, particularly those in the Philippines where founder populations were sometimes extremely small, is often caused primarily by the introgression of another species (e.g. Taniguchi *et al.* 1985; Macaranas *et al.* 1986). The presence of alleles and RAPD markers which were not detected in samples of wild species, may also be due to intragenic recombination in hybrids or elevated mutation rates (Clarke 1968; Barton *et al.* 1983). However, there are several other reasons why these alleles were not detected in wild populations, including the inadequate sampling of wild populations, genetic drift and the presence of non-endemic species in farm pond populations. Furthermore, alleles not maintained in the wild populations may continue to exist in farm pond populations due to their selection under the different environmental conditions. The higher levels of genetic variation observed in farm pond populations were also supported by significantly greater morphological variation and the morphological distinctness of many hybrid individuals. Unique morphologies in farm pond fish may also be caused to some extent by environmental effects, the presence of exotic *Oreochromis* species (not represented in the analyses) and insufficient sampling of the wild population.

6.2.3 The implications of hybridization on farm pond populations in Malawi

Hybridization in many cultured tilapia stocks has largely been the result of incorrect species identification, which is further complicated by the presence of hybrids (McAndrew & Majumdar 1983). Morphological characters are presumably the only agents used to identify fish when stocking ponds in Malawi and, as shown in this study, are reliable when distinguishing between *O. shiranus* and chambo. The identification of chambo species requires knowledge of their distinguishing features, care and experience, and is more reliable when identifying sexually mature

fish. Wild *Oreochromis* fingerlings from the wild, which are virtually impossible to identify, are often used to stock ponds. In fact, in a study of the culture performance of *O. karongae* in polyculture with *Clarias gariepinus,* fingerlings that were obtained by beach seining proved impossible to identify and were therefore collectively referred to as chambo (Maluwa *et al.* 1995) Furthermore, Chikafumbwa *et al.* (1997) reported that local communities plan to get fingerlings from the wild for stocking, but there was no mention of species identification and the problems of identifying juveniles. If fish were effectively identified during stocking of the ponds at Domasi and Mzuzu, then the mixing of chambo (*O. karongae*?) and *O. shiranus* stocks has presumably occurred afterwards. Possibly mechanisms for the mixing of stocks include escape through flooding or irrigation, the activities of predators (otters and fish eagles) and poor stock management where fish are thrown back into the wrong ponds.

As the aquaculture of *Oreochromis* species in Malawi expands, it is likely that farm fish of uncertain species identity and genetic purity will be distributed to many small-holder ponds. The distribution of impure tilapia stocks to fish farmers throughout the Philippines may have been a contributing factor to the frequent occurrence of introgressed hybrid populations (Taniguchi *et al.* 1985). Furthermore, the distribution of Indian major carp hybrids throughout India, that were produced inadvertently in commercial hatcheries, threatens the viability of commercial populations and the genetic integrity of native populations (Padhi & Mandal 1997). In Malawi, farm fish from Domasi have been used to stock small water bodies such as reservoirs and have been distributed to other farm ponds. It is highly likely that the stocking of many small-holder ponds throughout Malawi has gone undocumented.

Employees at the fish farms sampled in Malawi reported no change in yields or fitness since stocks have become mixed, although questions were often not answered due to a lack of knowledge. Nevertheless, it seems unlikely that hybridization between the culture species will be beneficial. Hybrids are often favoured in aquaculture since heterosis can be observed for growth, food conversion and disease resistance (Schonewald-Cox *et al.* 1983). The uncontrolled mixing and breeding of fish, as observed in Malawian farm ponds, is unlikely to produce populations with stable hybrid performance traits because few fish will obtain the maximum benefit of F_1 hybrids. Before the production of commercial stocks through genetic manipulation and hormonal sex-reversal (Mair & Little 1991; Mair *et al.* 1992, 1997), the benefits associated with hybrids were only realised through their controlled and deliberate production. For example, the beneficial cross between *O. niloticus* and *O. aureus* to produce nearly all-male progeny, is unidirectional and

reciprocal crosses and backcrosses have to be eliminated by thorough segregation of broodstocks (Taniguchi *et al.* 1985; Wohlfarth 1988). If hybridization between species in Malawi farm ponds results in unusual sex ratios, this may result in reduced population growth if there is a deficit of females. However, fish in the farm ponds sampled appeared to be breeding prodigiously. Alternatively, an excess of hybrid males may encourage back-crossing, particularly if they are more competitively aggressive than pure-bred males for resources including spawning ground.

Although F_1 hybrids may initially exhibit heterosis, the subsequent generations often display a decline (or reduction) in fitness due to outbreeding depression (Templeton 1986). Outbreeding depression, possibly due to chromosomal differences or the disruption of coadapted gene complexes (Templeton 1986; Carvalho 1993), may also be expressed in first generation hybrids. Hybrid inviability is not generally considered adaptive and is believed to evolve as a pleiotropic consequence of other genetic changes between diverging populations (Johnson & Wade 1995). The inherent instability of introgressed populations often results in variable and uneconomic hybrids (Eknath *et al.* 1991). If not already observed, it is likely that the introgressed populations in Malawian farm ponds will display a decline in fitness and most importantly a decline in growth performance. Most of the remaining *O. niloticus* in Lake Itasy, after hybridizing with and eliminating *O. macrochir,* were found to have a poor growth rate (Daget & Moreau 1981). Furthermore, the introgression of *O. mossambicus* genes into cultured *O. niloticus* populations in the Philippines, is thought to be a contributing factor to the poor or variable growth performance observed in these populations (Macaranas *et al.* 1986).

In cultured populations that have a history of hybridization it is often difficult to monitor and detect genetic changes due to genetic drift, inbreeding and the effects of natural and artificial selection. For example, levels of genetic variability may be deceptively high due to the introgression of another species (Taniguchi *et al.* 1985; Macaranas *et al.* 1986). If the aquaculture of *Oreochromis* species is to become a commercially viable and sustainable industry in Malawi, than it is important that pure species are maintained for fish breeding and stock improvement work (Eknath *et al.* 1991). Taniguchi *et al.* (1985), in a study of Philippine aquaculture, recommended that new founder stocks of commercial tilapia should be established and monitored regularly for distribution to fish-seed suppliers wishing to upgrade their broodstocks. It would be a major setback to the development of aquaculture in Malawi if stocks are not monitored initially, when it would be possible to avoid, or at least, minimise any decline in culture performance and the widespread distribution of impure stock.

6.2.4 The implications of escaped farm fish on the wild populations in Malawi

The cichlid species flocks of Lake Malawi are characterised by extremely high diversity (over 600 species) and marked endemism (99% endemic) (Konings 1989; Turner 1997; Martens 1997). Lake Malawi contains not only more species of fish, but also a wider variety of different trophic adaptations than all of the fresh waters of Europe combined (Turner 1995). Such mature lake communities are very susceptible to any changes. This is illustrated by the rapid loss of genetic diversity in Lake Victoria caused by the combined affects of species introductions (Ogutu-Ohwayo & Hecky 1991), destructive fishing practices (e.g. overfishing and the removal of nests by beach seining) and other man-made ecological changes (e.g. increased sedimentation and eutrophication). The widespread escape of cultured fish into the wild could have serious evolutionary consequences for the *Oreochromis* species of Lake Malawi, disrupting the unique chambo species flock. This species flock is the only case of intralacustrine speciation by tilapias in the presence of competing haplochromine cichlids. Of as much concern is the escape of the non-endemic species *O. mossambicus* from farm ponds and reservoirs. A full consideration of the implications of hybridization not only includes the impact that escaped hybrid fish may have on wild populations, but also the impact of hybridization between wild and cultured fish.

6.2.4.1 The escape of farm fish

Avenues for the escape of farm fish were apparent at all the sites sampled in this study, although at present, there is no evidence to support this process of contamination. The risks of escape are greatly increased during the rainy season when fish ponds are flooded. In fact, flooding often results in the need for the complete renewal of fish stocks in Malawian farm ponds (Chimatiro & Janke 1994). There is little documented evidence of *Oreochromis* species escaping from sites of aquaculture, although it is often suspected. Schwanck (1995) reported on the escape of *O. niloticus* from fish farms onto the Kafue flood plain in Zambia, although in this case there is no evidence of hybridization with local tilapias. Gregg *et al.* (1998) suspected that hybrids between the *Oreochromis* species of Zimbabwe (not naturally sympatric) could escape from artificial lakes into native river systems. Hybridization between *Oreochromis* species in natural waters have usually been through accidental introductions, often involving the inaccurate identification of species, or through intentional introductions. Nevertheless, in many cases such as the feral populations of tilapia in Australia, it is not known whether fish escaped or were introduced (Mather & Arthington 1991). The impact of escapees is partly dependant on the

numbers that escape, but as aquaculture increases in Malawi, the escape of cultured fish will become more likely.

6.2.4.2 The viability of farm fish in the wild

Apart from the numbers of fish that escape, the impact of escaped farm fish on wild populations is also dependent on their viability in the wild. If relatively few fish escape, which have a reduced fitness and reproductive potential compared to wild individuals, then their impact on wild populations may be minimal. Nevertheless, since stocks of chambo have declined in recent years due to overfishing (Turner 1995), competition between farm fish and wild fish for resources may be low. Inbred cultured fish populations, which have reduced heterozygosity, often exhibit a depression of fitness traits, such as growth, survival and fecundity (Kincaid 1983; Allendorf & Leary 1986; Gall 1987; Leberg 1990; Walman & McKinnon 1993), including tilapia stocks where few founders are associated with a loss of genetic variation through genetic drift (Brummett *et al.* 1988; Hulata *et al.* 1988; Pullin & Capili 1990). If the farm pond populations in Malawi have reduced fitness traits, the fish may have reduced competitive ability among wild populations. Farmed fish may have diverged from their wild conspecifics through local selective pressures in the pond environment, and may thereby be less adapted to the wild. Some of the farm pond populations studied had alleles which were not found in the wild populations, which may suggest that they are under different selection pressures, or that genetic drift has occurred. However, these alleles may not have been detected in the wild populations due to inadequate sampling.

Farmed Atlantic salmon (*Salmo salar*) have been found to be more aggressive than fish from natural populations, although less responsive to predation risk and reproductively inferior. Fleming *et al.* (1996) found that males exhibited inappropriate mating behaviour and females displayed less breeding behaviour. Nevertheless, interbreeding between cultured and wild salmon is common, and the offspring have been found to exhibit hybrid vigour (e.g. Ferguson *et al.* 1988; Einum & Fleming 1997). Alternatively, the hybrids may exhibit an array of traits maladaptive to the local environment as a result of the disruption of local adapted gene pools and their associated adapted gene complexes (Carvalho 1993). In fish, outbreeding depression (e.g. Lachance & Magnan 1990) seems to be more common than hybrid vigour (Einum & Fleming 1997) (implications discussed in section 6.2.4.3). Since interspecific *Oreochromis* hybrids often exhibit increased vigour and enhanced reproductive potential (Lovshin & De Silva 1975; Wohlfarth & Hulata 1981), the escape of relatively few hybrid fish from farm ponds in Malawi may have an

affect on wild populations. Hybrids may be more aggressive and have better competitive abilities than wild species for resources including spawning sites. However, since the hybrids found in this study were of post-F_1 generation they are unlikely to have a higher fitness than the parental species. Furthermore, hybrids have often disappeared, after being relatively common, in cases where hybridization has occurred between *Oreochromis* species in the wild following an introduction (Moreau 1983). Nevertheless, even if hybrids have the ability to survive, it is unlikely that a population of farm pond hybrids will be maintained in the wild. Firstly, because of the relative scarcity of hybrid mates in the wild and secondly, because hybrids may have a mate preference for the parental species (based on the study of mate choice; Chapter 2). Therefore, the impact of escaped farm pond hybrids depends on the extent of breeding with wild populations and the viability of the offspring.

There are several factors which may prevent the interbreeding of farm pond hybrids and parental species in the wild. Even if hybrid males have the competitive ability to obtain spawning sites they may not be successful at attracting mates. Under natural conditions, where fish are not crowded, water clarity is good and there is no shortage of conspecific mates (unlike the farm pond environment), mechanisms that incite interspecific recognition may be exacting and thereby completely prevent hybrids from breeding with the parental species. Hybrid males may not build attractive nests, their courtship behaviour may not be appropriate or their colouration may not be attractive to females. Nest form varies little between chambo species and it is not thought to be important in aiding mate recognition (Turner & Robinson 1991; Turner *et. al* 1991b), although nest shape does vary between chambo and *O. shiranus*. The characteristic chambo nest consist of a crater within which is built an elevated platform (McKaye & Stauffer 1988), whereas *O. shiranus* nests are basin-shaped (Trewavas 1983). Crapon de Caprona & Fritzsch (1984), in a study of hybrids between *Haplochromis burtoni* and *H. nubilus,* found that F_1 males built a 'sloppy' version of the *H. nubilus* nest. Hybrid males may build nests which are not attractive to either *O. shiranus* or chambo females. The breeding colours of *O. shiranus* are different from those of chambo, and may play an important role in maintaining reproductive isolation between these species. Furthermore, *O. shiranus* males do not have the characteristic genital tassel of chambo. Hybrids which exhibit a combination of colours may not be attractive to either parental species when there are conspecific mates available. Nevertheless, the later generation backcross hybrids found in farm ponds often resembled one of the parental species in colouration, and may also have a similar behaviour. These individuals may not be at such a disadvantage at attracting mates in the wild as

F_1 hybrids. Differences in the breeding colouration of chambo species are much more subtle. The appearance of hybrids between these species, particular between *O. karongae* and *O. lidole*, may not differ greatly from the parental species. However, since behavioural and chemical cues may be important to intraspecific identification, and thereby responsible for the continued separation of the species (Turner *et al.* 1991b), hybrids which do not exhibit the exact signals may not be successful in attracting mates. Nevertheless, hybrid females may not be at such a disadvantage in finding mates because of the higher reproductive rates and thereby lower 'choosiness' of males.

6.2.4.3 The detrimental impacts of escaped farm fish on wild Oreochromis *populations*

There have been no other documented cases of hybridization between naturally sympatric species of *Oreochromis* in aquaculture within their native country. Furthermore, there have been few reports on the impact of escaped cultured (pure or hybrid) *Oreochromis* species on natural populations. Great efforts have been made to document tilapia introductions (e.g. de Moor & Bruton 1988; Welcomme 1988; Agustin *et al.* 1996), but there are large gaps of knowledge regarding their environmental consequences. Reports on the impact of hybridization between *Oreochromis* species following introductions, and on the effects of escaped cultured fish of other species such as salmonids on natural populations, may provide an insight into the impact of escaped farm pond fish on wild populations in Malawi. The implications of the escape of hybrids between *O. shiranus* and *O. karongae* will be considered first, but the effects can be applied to an incidence of hybridization between any of the *Oreochromis* species of Lake Malawi. Similar effects can also be expected if *O. mossambicus* escape from farm ponds.

Since farm pond hybrids are most likely to be hybrids between *O. karongae* and *O. shiranus* (these are the species most commonly farmed), it will probably be these species which are immediately affected by the escape of hybrids. Hybrids (either directly from farm ponds or from interbreeding in the wild) may compete aggressively with the parental species and other native species for resources, such as food and spawning sites. The escape of *O. mossambicus* from farm ponds, could similarly result in increased competition. The availability of suitable spawning sites is thought to be one of the most important factors limiting populations (Welcomme 1966; de Moor & Bruton 1988). Nevertheless, resources utilized by chambo may not be limited due to the resent decline in stocks. Hybrids may be able to utilize both spawning sites of the parental species. *O. shiranus* generally remains in shallow vegetated areas throughout its life, breeding inshore (to 4 m deep) in sand or mud-banks near reeds (Trewavas 1983). Its breeding season overlaps with that

of chambo, which breed in a variety of substrata, including mud, sand and rocky beaches. *O. karongae* is the most inshore living of chambo species, breeding in water from 0.5 to at least 28 m (Turner *et al.* 1991b), and like *O. shiranus* is mainly a benthic-feeder. It has often been difficult to separate the effects of hybridization from competition following the introduction of *Oreochromis* species into the range an established species (Ogutu-Ohwayo & Hecky 1991).

The long-term effects of the introduction of hybrids into the wild populations is unknown, although in evolutionary terms any species changes may occur extremely rapidly. For example, after the introduction *O. niloticus* into Lake Victoria in the early 1950s, it took just over tens years for hybrids of *O. niloticus* and *O. esculentus* to become common (Welcomme 1966). By the 1980s, *O. niloticus* had contributed to the elimination of the native species (*O. esculentus* and *O. variabilis*) and dominated the tilapiine fishery (Ogutu-Ohwayo & Hecky 1991). Determining the effects of hybrids on wild populations will be complicated if the escape of farm fish is sporadic, occurring mainly during the rainy season. In general, there are two possible outcomes from the interbreeding of wild and escaped hybrid fish: i) the introgression of *O. shiranus* genes into the *O. karongae* population or visa versa, but the two species remain intact and reproductively isolated; ii) a breakdown in the mechanisms which allow for reproductive isolation between *O. shiranus* and *O. karongae*, and ensuing hybridization between the species. Persistent hybridization could result in the elimination of one or both species, as has commonly been observed after hybridization events following the introductions of non-native species (e.g. Daget & Moreau 1981; Ogutu-Ohwayo & Hecky 1991; Leary *et al.* 1993). Nevertheless, factors causing the elimination of a species are complex and unlikely to be due to a single process such as hybridization.

In both scenarios outlined above, introgressive hybridization could lead to a decline in the performance of wild populations through outbreeding depression. This could have implications both for the diversity of *Oreochromis* in Lake Malawi, and the viability of remaining *Oreochromis* fishery. Genetic introgression is considered to be nearly irreversible, and the final consequence can be the extinction of the taxon (Rhymer & Simberloff 1996; Berrebi 1997). A decline of native species has frequently been observed following the introduction of non-native forms, particularly in species of trout (e.g. Leary *et al.* 1993; Campton & Johnston 1985). Introgressive hybridization is thought to be a contributing cause to the decline of many western trouts as distinctive forms (Busack & Gall 1981). Hybridization is also likely to lead to a reduction in the genetic variability of natural populations (Carvalho & Hauser 1995), and may result in the elimination of unique genotypes. In turn, the unique qualities of the native species may be lost (Daget & Moreau 1981;

Dowling & Childs 1992; Berrebi 1997). In fact, introgressive hybridization in many cultured tilapiine species has already resulted in such extensive mixing of gene pools that many "pure" species may have been lost (FAO/CIFA 1985; Carvalho & Hauser 1995).

A change in the genetic composition of *O. karongae* populations may have an immediate affect on the other chambo species of Lake Malawi due to their close genetic relatedness and similarity in ecological requirements. Such changes could form a route for the breakdown in existing isolation barriers. Mechanisms which maintain pre-zygotic isolation may be similarly disturbed if farm pond hybrids between chambo species escape and interbreed with either parental species in the wild. Chambo show no spatial segregation of breeding grounds, breeding seasons overlap and diets are very similar (Turner & Robinson 1991; Turner *et al.* 1991a, b). The factors that maintain reproductive isolation between these species, are likely to be particularly fragile and susceptible to change. The ultimate consequence may be the loss of one or more of these unique species.

Interbreeding between farmed and wild fish may also result in a decline in the performance of natural populations through the disruption of local adaptations (Templeton 1986; Carvalho 1993; Rhymer & Simberloff 1996). The introduction of genetic novelty through interbreeding can result in a breakdown of the coadapted gene complexes associated with local adaptation. Such outbreeding depression has been well documented in salmonid populations, where local adaptations are particularly prevalent and threatened by the escape and introduction of farmed fish (Hindar *et al.* 1991; Heggberget *et al.* 1993; Einum & Fleming 1997). Local adaptation has not been well documented in tilapiine populations. However, the considerable habitat related geographic variation in morphological traits (particularly of the pharyngeal bone) found in *O. karongae* (Turner & Robinson 1991) may be evidence of local adaptation. Geographic variation has also been observed in most of the external and pharyngeal measurements of *O. squamipinnis.* Turner *et al.* (1991) suggested that the morphological differences between species (and populations) may be a phenotypic response to the dominant diet experienced in different habitats. However, it is not known whether these varying characteristics have a genetic basis. These 'local adaptations' may allow for niche separation between species and may thereby be an essential component in maintaining species biodiversity.

A wide variety of outcomes, from no detectable effect to complete introgression or displacement, has been observed following releases of cultured fish into natural populations (Hindar *et al.* 1991). Hybrids between cultured and wild fish often have reduced fitness traits, such

as a decreased survival of fingerlings and increased susceptibility to disease, as compared to indigenous fish (Hindar *et al.* 1991; Carvalho 1993). Hybridization between wild and cultured fish leads to a dilution of locally adapted gene pools, which in turn can lead to a reduction in population performance (Skaala *et al.* 1990; Hinder *et al.* 1991; Carvalho 1993). Interbreeding between farm strains, which often show a reduction in heterozygosity compared to wild populations, may also result in a reduction of genetic variability in wild populations (Clifford *et al.* 1998). A reduction in genetic variability may reduce the long-term adaptability of individuals, and ability to adapt to environmental change (Allendorf & Leary 1986; Soulé 1986; Utter *et al.* 1993; Rhymer & Simberloff 1996). The preservation of genetic variability is particularly pertinent in a time of rapid human-induced environmental change (Lynch 1996). Furthermore, the destruction of coadapted gene complexes in local populations is as irreversible as the loss of alleles or a species (Carvalho 1993). Since offspring fitness declines as the genetic differences between putative parents increases (Templeton 1986; empirical evidence reviewed in Carvalho 1993), the detrimental effects of interbreeding between cultured and wild populations will be dependent of how genetically different they are. If the aquaculture of native *Oreochromis* species becomes more popular and commercialised in Malawi, farm stocks may become increasingly diverged from their wild conspecifics through intended and unintended artificial selection. The use of domesticated strains of *O. shiranus* is already widespread. In the future, the escape of cultured fish may become more of a threat to the genetic integrity of wild populations.

6.2.4.4 The impact of escaped farm fish on the Lake Malawi ecosystem

The escape of farm fish, and the resulting hybridization with wild populations, may affect the other endemic species of Lake Malawi in several ways, although it is difficult to predict the outcome of such changes. Nevertheless, in such a complex lake ecosystem slight changes in species composition are likely to have an effect. Firstly, there may be increased competition for resources. Hybrid genotypes constitute a source of novel genetic diversity, that can have new ecological tolerances and thereby the adaptive potential for colonizing environments not occupied by either parental population. In Australia, for example, the introduction of two strains of the common carp (*Cyprinus carpio*) gave rise to a new, more vigorous and ecologically more tolerant strain, which became far more widespread and problematic than the parental stocks (Arthington 1991). Similarly, hybrids between the *Oreochromis* species in Lake Malawi may utilise habitats which were previously occupied by other species. This may lead to an increase in competition for

food, space and breeding areas, and eventually the competitive exclusion of the affected species.

Alternatively, it may be a loss in *Oreochromis* species diversity which leads to a change in the structure of the Lake Malawi community. Other benthic and plankton feeding species may fill a vacant niche. Assessing the impact of escaped farm fish may be complicated by the changes in fishing practices. Fishing, of the intensity found in Lake Malombe and in Lake Malawi south of Boadzulu Island, has led to great changes in community structures, with a reduction in diversity and increasing dominance of smaller species. The chambo dominated fisheries has now been replaced by the smaller demersal and pelagic haplochromine cichlids (Turner 1995). In Lake Victoria a loss of genetic diversity, associated with the introduction of *O. niloticus* and the Nile perch *Lates niloticus,* has been accompanied by a loss of trophic diversity. The transformation of the fish community coincided with significant eutrophication, which might be related to alterations of the lake's food-web structure (Ogutu-Ohwayo & Hecky 1991).

6.2.5 Recommendations and further research

To avoid the deleterious consequence of hybridization between species in aquaculture, the correct identification of species prior to stocking is crucial. Preferably, this would involve the use of molecular genetic techniques to confirm unequivocally the identity of species. Further research is required to identify genetic markers of chambo species (see section 6.2.2.1). Alternatively, if genetic markers for chambo species prove to be elusive, stocks of *O. karongae* should be established using sexually mature adults taken from the wild which have been identified by experienced persons. The stocking of ponds with unidentified fingerlings collected from the wild is not recommended. In a country where financial resources are limited, and the facilities and expertise needed for genetic analysis are rare, using morphological characters for species and hybrid identification may be the only option to many farmers. In the present study, where the author and co-workers were relatively inexperienced at species identification, the multivariate analysis of morphological variables suggests that only one sexually mature chambo fish had been incorrectly identified. Furthermore, morphological characters are valuable in providing initial evidence for the presence of hybrids (between chambo and *O. shiranus*). Nevertheless, it would be difficult to encourage farmers, who are only interested in a reliable source of food, to be so circumspect about the purity of their fish stocks.

When the identity of farm fish has been verified, the systematic elimination of contaminated stocks would be advisable. It would be expensive and impractical to test for the

identity of every fish using genetic techniques, and the use of morphological characteristics would be unreliable where hybrid individuals resemble a parental species. Furthermore, if extensive introgression has occurred hybrid individuals may also have genotypes resembling a parental species. In the Philippines some farmers unsuccessfully attempted to clean their *O. niloticus* stocks by removing and destroying fish which resembled *O. mossambicus* (Taniguchi *et al.* 1985). Therefore, the complete elimination of a contaminated farm stock is recommended. However, this strategy is likely to be unpopular, particularly with small-hold farmers who do not care about the purity of their stocks and have insufficient funds to replace them. Therefore, risk analysis of potential problems arising from the stocking of fish of unknown origin is required before the elimination of stocks can be considered.

Management agencies often attempt to control introgression between native and introduced trout species by removing fish with hybrid characteristics (Busack & Gall 1981). However, extreme care must be exercised when considering the elimination of any contaminated wild population since the genetic identity of native taxon may be lost forever (Dowling & Childs 1992). Similar problems may befall the management of *Oreochromis* species in Lake Malawi, if the escape of hybrid fish from farm ponds forms a route for hybridization between natural populations. However, in recently diverged groups such as the *Barbus intermedius* complex of Lake Tana Ethiopia, persistent hybridization at low levels does not necessarily threaten the genetic integrity of the two parent groups (Nagelkerke & Sibbing 1996). Concern has been raised over conservation programmes which plan to protect endangered species with an active bias against hybrids. Such programmes may prevent an important evolutionary mechanism, and valid species of hybrid origin could be made extinct (DeMarais *et al.* 1992; Bullini 1994; O' Brien & Mayr 1991). Nevertheless, hybridization between *Oreochromis* species is usually the result of introductions by man and often results in the elimination of a species. Furthermore, there is little evidence to support hybridization as an important evolutionary mechanism in cichlids (Crapon de Caprona & Fritzsch 1984).

Once pure farm stocks are established, all necessary precautions should be taken to prevent the mixing of stocks. This would primarily involve the secure containment of fish, particularly during times when there is the risk of flooding. Nevertheless, in order to maintain the genetic integrity of *Oreochromis* species throughout sites of aquaculture in Malawi, regular monitoring of stocks for the presence of interspecific hybrids would be prudent. It is also necessary to ensure that cultured fish do not come into contact with natural populations since the potential loss to a complex lake ecosystem like Lake Malawi is too great. The cheapest strategy for the genetic

protection of native populations from the effects of aquaculture would involve secure containment. Again, this may be difficult to ensure during the rainy season, and impractical to expect farmers to be so conscientious. A more expensive, and therefore probably unrealistic option, which has been suggested for the protection of native salmonoid populations from domesticated stocks, is the use of sterilized fish (Hindar *et al.* 1991; Galbreath & Thorgaard 1995). In the field of transgenic tilapia, there has been recent research into the production of sterile fish through the introduction of a DNA sequence blocking production of one of the hormones necessary for reproduction (Roderick 1998).

Further research is required into the performance of farm (hybrid and pure) fish in the wild, so that effects of such introductions, and therefore the necessity for preventative action, can be realistically assessed. However, direct evidence for the escape of farm fish into Lake Malawi is a priority. One possible method to monitor the escape of farm fish is to label them. A variety of tagging or marking methods are available including injected dye and implanted telemetry transmitters (Parker *et al.* 1990; Wydoski & Emery 1983; Thoreau & Baras 1997). Implanted transmitters would provided detailed information on the movement of fish and would also be relatively easy to recover since the cooperation of fishermen would not be relied upon. However, fingerlings and juvenile fish are more likely to escape than adults, and would be more difficult to tag. Therefore, it may be more practical to genetically tag artificially produced fry. Genetic tagging would also enable the reproductive success of farm fish and gene introgression with wild fish to be monitored (Campton 1987; Utter & Seeb 1990; Jørstad *et al.* 1994).

The development of commercially attractive strains is essential for the advancement of aquaculture as a viable industry in Malawi. The use of readily available commercial strains of non-native species would probably be highly beneficial to yields, although it is essential that indigenous species continue to be used in aquaculture for the conservation of native populations. It is therefore important for the unique genetic resources of pure wild species to be maintained for selective breeding and stock improvement purposes (Pullin & Capili 1990). Confusion over the genetic status of *O. shiranus* subspecies (*O. sh. shiranus* and *O. sh. chilwae*) from Lakes Malombe, Chiuta and Chilwa, has resulted in the mixing of species and subsequent hybridization in aquaculture. Furthermore, *O. shiranus* strains which have desirable production characteristics cannot be ascribed with certainty to any species (Ambali *pers. comm.*). For the rational utilization and conservation of genetic resources, both in wild and cultured stocks, a full description of their genetic status is required (Eknath *et al.* 1991). Research into the amount and patterns of genetic

variation within and between populations of *Oreochromis* species in the wild could determine the level of population substructuring, which would have implications for fishery management. In the development of commercial strains, priority should be given to the importance of maintaining broodstock diversity, both for maintaining fitness in current stocks, and for the selective breeding of advantageous culture performance traits (Purdom 1993). Plans by the Malawian government and aquaculture industry for the genetic management of domesticated stocks should be implemented immediately, if they are not already in place. It would be beneficial if experimental fish farms and research centres to establish and breed commercial strains of tilapia which can be monitored regularly and distributed to commercial and small-holder fish farmers.

The development of aquaculture in Malawi, as outlined above, is heavily dependent on the financial resources available. It would be unfortunate if large commercial companies with financial backing are the only ones to benefit from aquaculture in Malawi. In Asia it is still mainly the well resourced private sector entrepreneurs and agribusiness that have adopted, and benefited from, technologies to produce all-male stocks. The public sector and small-scale hatcheries have largely failed to produce fish seed of a predictable and sustainable quality because of the relatively complex management and levels of investment required (Little 1998). The undernourished and rapidly expanding population of Malawi will benefit only if the development of aquaculture is supported at the community level. Small-holder fish farmers, with low resource input, tend to loose interest and abandon farming if returns are low (Maluwa 1994), and profits are only realised when fish farming is integrated with an existing livestock activity (Maluwa *et al.* 1995). It would be difficult to educate and make farmers appreciate the importance of good farming practices (such as maintaining brocdstock diversity, preventing the mixing and escape of fish) when they are poor and hungry. Pitcher (1995) reported that aquaculture it is unlikely to contribute more than 10% of the nations protein requirements. However, the current fish catch cannot keep pace with a large undernourished population growing at more than 3% per annum. Despite a local population crash of chambo in the SE Arm of Lake Malawi, the native tilapiines are not thought to be at immediate risk of extinction from overfishing because of their lake-wide distribution, large population sizes and their relatively large fecundity (Turner 1995). At most risk are the demersal and pelagic haplochromines, of which little is known. There is little local awareness of species diversity and fishing communities are unlikely to be interested in conservation. Species introductions into Lake Malawi are unlikely to increase yields or benefit the nation, and would probably drastically alter the ecosystem and bring about the collapse of most existing fisheries (Pitcher 1995). Therefore,

a dramatic increase in aquaculture with extensive government support appears to be one of the only options left for ensuring that protein is available for the nation of Malawi in the future.

6.3 SPECIATION IN THE *OREOCHROMIS (NYASALAPIA)* OF LAKE MALAWI

6.3.1 Speciation in sympatry?

The great majority of *Oreochromis* species are geographically isolated, and have evolved allopatrically. However, there are a number of factors which make it unlikely that the *Oreochromis (Nyasalapia)* of Lake Malawi are, or have been geographically isolated, which suggest that these species may have evolved in sympatry. Firstly, the three species are distributed throughout the lake and readily move offshore at certain times of the year (Turner & Robinson 1991). Major lake level fluctuations are thought to have contributed to the diversification of cichlids in the Great Lake of Africa (Sturmbauer & Meyer 1992; Martens 1997), although even at low water levels Lake Malawi may have only had one basin (Owen *et al.* 1990). The species flock exhibits a number of unique synapomorphic characters when compared with the other East and Central *Oreochromis (Nyasalapia)* species, it is thereby also unlikely that chambo have evolved through successive invasions by a riverine ancestral species or group of species (Turner & Robinson 1991). Whatever the speciation event was, the high levels of heterozygosity in all chambo species (this study and Sodsuk *et al.* 1995) suggest that it was not accompanied by a significant reduction in genetic variation. Moreover, this would imply that if caused by physical or behavioural isolation, large numbers of fish were involved, or if there was a bottleneck, the population must have expanded rapidly in size maintaining much of the genetic variation (Sodsuk *et al.* 1995). Sodsuk *et al.* (1995) estimated that the expected time since divergence ranges from 12-200 thousand years, suggesting that isolation was very recent.

The existence of sympatric speciation remains controversial (Mayr 1963; Paterson 1978; Bush 1994), although there are an increasing number of examples from a broad range of taxa where speciation has occurred in the absence of any apparent form of isolation. For example, cichlids within small crater lakes of Cameroon have speciated with negligible habitat patchiness (Schliewen *et al.* 1994) (although a degree of bathymetric segregation cannot be excluded; Martens 1997). The diverse pelagic species of the genus *Diplotaxodon* endemic to Lake Malawi, as well as chambo, do not appear to be restricted by habitat barriers (Turner 1994). Research on natural host races, comparative phylogenetic analysis, laboratory experiments and theoretical models has greatly strengthened the case for sympatric speciation (Bush 1994). Most models for sympatric speciation

assume strong disruptive natural selection leading to genetically based ecological diversification (Maynard Smith 1966, Thoday 1972; Bush 1994). In the presence of gene flow, between diverging populations, there may be selection for increased reproductive isolation where hybrids between two incipient species have reduced fitness (i.e. reinforcement) (Dobzhansky 1951; Butlin 1987, 1989). Fully reproductively isolated species may also evolve in sympatry by means of sexual selection (Lande 1982; Turner & Burrows 1995; Payne & Krakauer 1997).

Controversy remains over whether positive assortative mating can evolve through selection against the production of hybrids (Paterson 1978; Felsenstein 1981; Liou & Price 1994). Moreover, there is little empirical evidence where reinforcement has been demonstrated unequivocally (Butlin 1987, 1989; Butlin & Ritchie 1994). There has been no evidence to support reinforcement as an initial cause of colour divergence in cichlids (Deutsch 1997). Furthermore, hybrids between *Oreochromis* species do not generally exhibit considerable unfitness. The roles of ecological diversification and sexual selection as initial causes of divergence of *Oreochromis (Nyasalapia)* in sympatry will be considered with examples of these processes in other cichlid species, in particular the haplochromines.

Haplochromine cichlids are distinguished from tilapiines primarily on the basis of subtle differences in cranial and dental morphology (Trewavas 1983). The tilapiine tribe is believed to have diverged from ancestral haplochromine species more than 10 million years ago (Fryer & Iles 1972, Franck & Wright 1993). Many mechanisms have been proposed to explain the evolutionary success of cichlid species in the Great Lakes of Africa (e.g. Fryer & Iles 1972; Liem 1973; McKaye *et al.* 1984; Sturmbauer & Meyer 1992; Moran & Kornfield 1995; Seehausen *et al.* 1997; van Oppen *et al.* 1997). Almost all of the fish species that have originated in Lake Malawi over the past 0.5 million years are haplochromines. Molecular studies of haplochromine species have confirmed that speciation has occurred recently within the lake basins, and are not the result of repeated invasions by different ancestral lineages (Meyer *et al.* 1990; Kocher *et al.* 1993). Haplochromines possess several properties that favour rapid speciation by limiting intra-lacustrine dispersal (Martens 1997), including mouthbrooding behaviour, home-site fidelity (Hert 1992) and restricted habitat distribution (unlike chambo). The alternation of rocky and sandy shore habitats suggests that many of the inshore species may have arisen through allopatric speciation (Fryer & Iles 1972; Ribbink *et al.* 1983; van Oppen *et al.* 1997). Allopatric speciation might have been promoted by considerable fluctuation in the water levels of Lakes Malawi and Victoria (Owen *et al.* 1990;). Nevertheless, because habitats and niches are quite restricted for most of the species

and many species are found to inhabit individual rocky outcrops, sympatric speciation cannot be ruled out (Ribbink 1991; Galis & Metz 1998).

6.3.1.1 Ecological divergence

In a heterogenous environment, disruptive selection (i.e. selection favouring extreme at the expense of average phenotypes) can produce a stable polymorphism between alleles adapting individuals to different niches or habitats in the absence of an initial isolation by distance (Maynard Smith 1966). Adaptive traits, such as habitat preference, involved in the shift to a new niche are often the same traits that result in habitat specific assortative mating and the evolution of reproductive isolation between sympatric populations (Bush 1994). Lowe (1953) proposed that the *Oreochromis (Nyasalapia)* diverged sympatrically through separation of breeding sites or season. However, more recent studies have found great overlap between chambo species in breeding site (depth and substrate) and breeding season, and thereby provide no support for Lowe's theory (Turner & Robinson 1991; Turner *et al.* 1991b). Despite the overlap in the depth of breeding habitats, there is some restriction in distribution between species. For example, *O. lidole* nests have not generally been found at depths of less than 17 m (down to 50 m at least) and *O. squamipinnis* nests are not found at depths over 20 m (up to less than 1 m). However, the nesting habitat of *O. karongae* overlaps greatly with the other chambo, occurring at depths of 0.5 to at least 28 m (Turner & Robinson 1991). The possibility that chambo initially evolved through a divergence in feeding habitat preference will be considered.

Chambo form an ecological series showing increasingly specialisation to an open-water, plankton feeding existence which in turn is correlated with a progressive reduction of jaw and pharyngeal dentition. *O. lidole* is considered the most pelagic and most dependent on phytoplankton, while *O. karongae* (formerly *O. saka* (Lowe), Turner & Robinson 1991) and *O. squamipinnis* are believed to be more benthic in their diets (Lowe 1952; cited in Turner *et al.* 1991a). Ecological diversification may have been a key factor responsible for speciation of the monophyletic cichlids endemic to the crater lakes Barombi Mbo and Bermin crater in Cameroon (Schliewen *et al.* 1994). In Lake Bermin, for example, the two basal lineages of the flock (based on MtDNA analysis) separate the pelagic planktivorous species and substrate orientated feeders. Incidentally, the species that are either benthically or pelagically orientated in their feeding behaviour both breed close to the lake bottoms. Therefore, even if there were microgeographical sorting of subpopulations based on feeding behaviour, mating would still take place in sympatry

(Schliewen *et al.* 1994).

Nevertheless, different chambo species caught at the same place at the same time in the SE Arm of Lake Malawi have been found to have very similar diets (Turner & Robinson 1991; Turner *et al.* 1991a). Any avoidance of competition for food which occurs is likely to be due to habitat preference. Turner *et al.* (1991a) suggested that different chambo species may feed on the same food but from different microhabitats, explaining the variation found in the amount of sand in the stomachs of *O. lidole* and *O. squamipinnis*. Morphological differences observed between species (and populations) in feeding structure may be phenotypic responses to the dominant diet experienced in preferred habitats (Turner & Robinson 1991). Phenotypic plasticity (in morphology and behaviour) may contribute to speciation, either sympatrically or allopatrically, by allowing rapid shifts necessary for diversification (West-Eberhard 1989). The haplochromine cichlids of Lake Victoria show a striking diversity of feeding niches and sibling species are characterized by small differences in feeding behaviours. Liem (1973) considered that the flexible and versatile pharyngeal jaw apparatus of cichlids promotes evolutionary diversification by providing behavioural flexibility and evolvability. Nevertheless, closely related species of haplochromine differ mainly in male breeding coloration (Ribbink *et al.* 1983; Seehausen *et al.* 1997), not tooth morphology or mouth shape, and ecological studies have not found large differences in habitat or diet (Greenwood 1991; Ribbink *et al.* 1983). Therefore, the initial divergence of populations (in sympatry and allopatry) may not have been caused by adaptation to differing habitats. Based on the present knowledge of habitat distribution and diet in chambo, which overlap between all species, it seems unlikely that ecological diversification may have been the initial cause of divergence in sympatry.

6.3.1.2 Sexual selection

In the highly polygynous breeding system of maternal mouthbrooders, where females choose between competing males, sexual differences in body size and colour are prevalent (Fryer & Iles 1972; Trewavas 1983; Ribbink *et al.* 1983). Apart from being larger, more brightly coloured and more aggressive than females, *Oreochromis* males have many other features indicative of being strongly sexually selected. For example, males build spawning pits and develop secondary sexually structures such as exaggerated jaws and genital papillae. When males provide no resource or parental care and females can mate with numerous males, 'extravagant' male secondary sexual characteristics may result exclusively from sexual selection. Several authors have suggested that

local differences in female mate choice may have played a large role in the divergence of breeding colouration and speciation between African cichlids (e.g. Dominey 1984; McKaye *et al.* 1984; Deutsch 1997). Speciation through sexual selection may be a contributory mechanism in several other taxa which have undergone rapid speciation events, such as fruit flies and crickets (Dominey 1984). There are a number of theories as to how female choosiness may have arisen (Kirkpatrick & Ryan 1991; Andersson 1994), most commonly cited is the Fisherian runaway process, other reasons may be to secure strong and healthy mates which might carry genes for parasite resistance (Hamilton & Zuk 1982).

Population genetic models have shown that sexual selection could lead to rapid diversification of mate preference between allopatric (Lande 1981; Schluter & Price 1993) or parapatric populations (Lande 1982). More recently, in a simulation model, Turner & Burrows (1995) demonstrated that rapid speciation can occur in sympatry in species exhibiting sexual selection. Any reduction in gene flow is likely to increase the probability of speciation, so the mechanism proposed would also operate in allopatry, parapatry or where parts of the population were specialised to different habitats. Fully reproductively isolated species can evolve within a few generations, in a small population, on the basis of a single mutation in female mate preference rule and in the absence of disruptive natural selection. In contrast, under Lande's model (1982), sexual selection may have the effect of exaggerating variability of male traits along a cline where local differences in stabilizing natural selection have produced some geographic variability. Furthermore, full reproductive isolation was not established. In the model of Turner & Burrows (1995) any mutation leading to reversal of female preference rule may initially be aided in its invasion of the population by decreased mortality acting on the less handicapped males favoured by the mutant females. For example, highly conspicuously coloured males may suffer from high predation. After the establishment of reproductive isolation, competitive exclusion or character displacement can be expected to occur as further differences accumulate in the now separate gene pools. Under the biological species concept reproductive isolating mechanisms are viewed as a non-adaptive by-product of genetic divergence that develop between geographically separated populations (Avise 1994). In sympatric species that have evolved through mate preference, and in which it is mate choice that (initially) maintains reproductive isolation, Paterson's mate recognition concept (1980, 1985) may be more applicable. Under this concept species are defined in terms of recognition of conspecifics rather than of isolation from other species.

A change in female preference could be produced through a change in the sensory system

or neural system of the female which makes a different male display trait more conspicuous or attractive (Ryan & Rand 1993). Seehausen *et al.* (1997) suggested that variation in colour vision of haplochromines could explain why sympatric, closely related species usually have male colouration at opposite ends of the colour spectrum (blue and red/yellow). Small evolutionary changes can modify the maximum sensitivity of the eye from red/yellow to blue and vice versa. If females prefer conspicuous males, individual variation in colour vision could be responsible for the observed dichotomy in male colouration. It has been demonstrated that haplochromine cichlids choose mates within and across species on the basis of colouration (Turner *pers. comm.*; Hert 1992; Seehausen 1997; Seehausen & van Alphen 1998). Seehausen *et al.* (1997) reported that increased turbidity in Lake Victoria, which constrains colour vision, has resulted in dull fish colouration, few colour morphs and low species diversity. By interfering with mate choice, turbidity leads to relaxed sexual selection, preventing further speciation events, and blocks the mechanism of reproductive isolation thereby resulting in hybridization (Seehausen & van Alphen 1998).

Unlike the haplochromines, chambo do not differ greatly in male breeding colouration. This is particularly true for *O. lidole* and *O. karongae*, where breeding males are black. However, *O. karongae* males often have some bluish iridescent patches on the head or body and the flank scales often have a coppery metallic spot (Turner & Robinson 1991), although whether these subtle characters are fixed in this species is not known. Breeding *O. squamipinnis* males have paler bodies than the other chambo and have a uniquely pale head (or 'face mask') which varies in colour and can be blue, white or green. This species-specific characteristics may aid in species recognition and maintain reproductive isolation. However, whether male breeding colouration is sufficiently different between *O. lidole* and *O. karongae,* to suggest it is important in female mate preference is debatable. Furthermore, exactly how extreme males are naturally selected against (according to the model of Turner & Burrows (1995)), for example because adult chambo suffer from very little predation, is unknown. Nevertheless, colouration is not the only trait subject to female preference. Other sexually selected traits include nest form, courtship behaviour and secondary sexual structures such as genital papillae, all of which may be opposed by natural selection in their extreme forms because they are perhaps too energetically expensive.

Variation in bower form between sibling species of the genus *Tramitichromis* has been found to correlate with genetic distance measures, and is hypothesized to have arisen from nonadaptive differences in female choice (McKaye *et al.* 1993). McKaye & Stauffer (1988) suggested that nest form may be important in maintaining reproductive isolation between chambo

species, however no clear differences in nest form have been found (Turner & Robinson 1991; Turner *et. al.* 1991b). Dominey (1984) proposed that sexual selection acting on sexual behaviour may be an important force in species flock formation. However, courtship behaviour shown by male mbuna has been found to be conservative in pattern and sequence, and there has been no evidence to suggest that female choice operating on male courtship behaviour has been a major force in the radiation of haplochromines (McElroy & Kornfield 1990). Courtship behaviour between species of *Oreochromis* has been found to vary little (present study, Chapter 2; Elder *et al.* 1971; Falter & Dufayt 1991). Since there have been no behavioural studies on chambo, the possibility that female choice for behavioural cues may be responsible for maintaining reproductive isolation cannot be ruled out. Female choice for chemical cues may also be important. In fact, the association of male and female *O. lidole* in small groups late in the day when courtship does not take place, suggests that species recognition occurs in the absence of cues associated with breeding (such as breeding depth and season, nest form and male colouration) (Turner *et al.* 1991b). Mate choice experiments would be extremely valuable in elucidating how chambo species recognise conspecifics and select mates, and thereby determine the traits on which sexual selection may act.

6.3.1.3 Concluding remarks

Based on the present knowledge of the ecology and behaviour of chambo it is not possible to determine precisely how these species evolved and how they maintain reproductive isolation. Isolation between chambo species may result from the combined effects of several partially isolating factors, each of which alone is insufficient (Elder *et al.* 1971). Similarly, their speciation may be the combined effect of several processes. Galis & Metz (1998) stated that it is essential to combine insights from different disciplines when analysing sympatric and parapatric speciation processes. In the case of the haplochromine cichlids of Lake Victoria, Galis & Metz (1998) proposed the following scenario. Sexual selection for strikingly coloured males is the driving force behind isolation of colour morphs (Seehausen *et al.* 1997). Disruptive selection on feeding and other specializations (adaptive radiation) promotes the divergence of these incipient species and the resulting niche shifts promotes their coexistence (Liem 1973).

In chambo, sexual selection may be acting on other traits apart from colouration, and it may be different traits in each species. For example, in *O. squamipinnis* it may be male breeding colouration, since this differs greatly from the other chambo species. Whereas, in *O. karongae* and *O. lidole* it may be courtship behaviour. If many of the haplochromines evolved due to a change

in female preference (Turner & Burrows 1995; Seehausen *et al.* 1997), the lack of diversity in chambo may be due to a lack of change in female preference, in that, the chambo genome perhaps has a lower mutation rate or population size than the haplochromines. There are several other explanations as to why chambo are not as diverse as the haplochromines in Lake Malawi. One possibility concerns the adaptive radiation of haplochromines. The flexible pharyngeal jaw apparatus of cichlids, which allows rapid diversification, can explain why haplochromines of Lake Victoria display such diverse adaptive radiation (Liem 1973; Galis & Metz 1998). Since tilapiines are distinguished from haplochromines by subtle differences in cranial and dental morphology (Trewavas 1983), there must be some reason as to why they do not display similar feeding diversity. Perhaps the jaw apparatus of tilapiines is not as flexible as that of haplochromines, or that feeding behaviour is not as plastic. Nevertheless, Trewavas (1983) suggested that not only is it unlikely that the chambo species, with dental specializations for planktivorous feeding, would revert to insectivory or aspire to mollusc-eating, but the shore of Lake Malawi is crowded with *Haplochromis* species that have preempted these niches.

Finally, the lack of geographical restriction in chambo is probably a major factor in reducing the probability of speciation. Most mbuna are endemic to only one particular island or geographically restricted to parts of the rocky shore in Lake Malawi. The patchy distribution of inshore habitat, in conjunction with the lack of dispersal due to mouthbrooding behaviour, homing ability and home-site fidelity (Hert 1992), promotes the chances of allopatric speciation (Fryer & Iles 1972; Ribbink *et al.* 1983; McKaye *et al.* 1984). Recent research, in which microsatellites were used to examine genetic differentiation between subpopulations on adjacent headlands, suggested that population subdivision in mbuna may be on a finer scale than previously thought (van Oppen *et al.* 1997). The division of fish populations into thousands of subunits may provide unprecedented opportunities for allopatric speciation (van Oppen *et al.* 1997).

6.4 SUMMARY AND CONCLUSION

The finding that hybrids of *O. shiranus* and chambo are present in farm ponds is of significant concern to the aquacultural industry of Malawi, and to the captive fisheries because of the possible consequences of escapees on the unique fish species flocks of Lake Malawi. As the population of Malawi proceeds to grow, the already overexploited cichlid fisheries is unlikely to keep pace, and the aquaculture of tilapiines may become an increasingly necessary option. Uncontrolled hybridization between cultured species is unlikely to be beneficial. The elimination

of contaminated stocks is advisable to prevent the distribution of fish of uncertain species identity and genetic purity. The risk of cultured fish escaping into the wild is also likely to increase as aquaculture expands. Introgressive hybridization between wild and cultured fish could lead to a decline in the performance of wild populations, affecting both the diversity of *Oreochromis* and the viability of the remaining *Oreochromis* fishery. Such species changes could have detrimental consequences on the other native fish species of Lake Malawi. The introduction of commercial strains of exotic species may pose an even greater threat to the native species. It is therefore essential that the unique genetic resources of pure wild species are maintained for selective breeding and stock improvement purposes, and that studies on the genetic structure of wild and farm pond populations are carried out. The development of aquaculture in Malawi is heavily dependent on the financial resources available and requires full government support.

Several options for future research have been identified, these include:

1) Further examination of farm pond stocks to determine if hybridization is occurring between chambo species. Highly polymorphic microsatellite loci may have great potential for revealing genetic variation between the chambo species.

2) Assessments on the extent to which cultured fish escape into the wild, on the viability of cultured fish in the wild, and on the impact that cultured fish could have on wild populations. This requires a full description of the genetic status of both wild and cultured stocks.

3) Mate choice and ecological studies are required to gain a better understanding of how reproductive isolation evolved and is maintained between the *Oreochromis (Nyasalapia)* of Lake Malawi.

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APPENDIX I: MATE CHOICE

Table I.1 Mean time (± SE) that females spent in front of either male in Open-choice experiments, where the female had a choice of two different male types (three replicates, each experiment 30 minutes long) and the results of a paired *t*-test for each experiment.

Female type	Two males present	Mean time with each male (s / 30 minutes)	Longest time*	*t*-value	*P*
OMOSS	OMOSS	1183.0 ± 140.7	3	2.47	0.132
	OSPIL	522.0 ± 129.5	0		
	OMOSS	937.7 ± 230.4	2	0.44	0.706
	HYBRID	738.3 ± 235.4	1		
	OSPIL	725.0 ± 148.2	1	-0.94	0.448
	HYBRID	1005.7 ± 151.6	2		
OSPIL	OMOSS	473.0 ± 27.62	0	-15.45	0.004
	OSPIL	1159.3 ± 17.34	3		
	OMOSS	631.3 ± 215.9	0	-1.09	0.391
	HYBRID	1039.3 ± 163.1	3		
	OSPIL	1014.7 ± 121.1	2	1.24	0.341
	HYBRID	635.0 ± 146.4	1		
HYBRID	OMOSS	918.7 ± 177.0	3	2.30	0.148
	OSPIL	648.0 ± 153.2	0		
	OMOSS	597.7 ± 189.0	1	-1.31	0.321
	HYBRID	1042.3 ± 154.4	2		
	OSPIL	556.7 ± 106.0	0	-1.55	0.262
	HYBRID	1040.3 ± 223.7	3		

* Number of times that each male has the longest time spent with it, out of three replicates.
OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

Table I.2 Mean (± SE) number of displays performed by females, for six behaviours recorded during Forced-choice experiments (nine replicates of each combination of female type and male type).

Female	Beh.*	Male type		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	21.0 ± 6.6	10.4 ± 4.1	31.4 ± 16.9
	Di.	3.1 ± 1.4	8.9 ± 5.2	6.3 ± 4.2
	F.D.	9.0 ± 4.0	5.1 ± 1.4	9.7 ± 3.2
	L.D.	12.7 ± 2.4	9.7 ± 2.3	13.0 ± 2.7
	No.	3.3 ± 1.3	1.2 ± 0.6	3.1 ± 0.9
	Sh.	2.2 ± 1.1	1.9 ± 1.3	0.4 ± 0.2
OSPIL	Bi.	13.8 ± 4.2	10.8 ± 4.0	27.9 ± 14.6
	Di.	2.8 ± 1.2	6.2 ± 2.7	8.8 ± 5.6
	F.D.	10.6 ± 2.9	10.0 ± 2.9	16.2 ± 5.2
	L.D.	8.7 ± 2.2	10.4 ± 1.9	14.8 ± 2.9
	No.	1.8 ± 0.9	1.7 ± 0.7	1.7 ± 0.6
	Sh.	0.2 ± 0.1	1.7 ± 0.7	1.4 ± 0.7
HYBRID	Bi.	61.0 ± 22.4	18.7 ± 5.0	60.7 ± 22.1
	Di.	18.8 ± 5.7	35.2 ± 10.3	10.1 ± 4.2
	F.D.	9.2 ± 4.4	9.1 ± 3.4	11.8 ± 3.6
	L.D.	11.4 ± 4.0	10.0 ± 3.0	15.4 ± 4.1
	No.	1.2 ± 1.2	2.0 ± 1.0	1.8 ± 0.8
	Sh.	3.7 ± 1.7	0.9 ± 0.6	3.3 ± 1.6

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

Table I.3 Mean (± SE) number of displays performed by females, for six behaviours recorded during Open-choice experiments, when both the males presented to the female are of the same type (three replicates).

Female	Beh.*	Both male types presented to female		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	17.3 ± 6.4	5.7 ± 2.0	6.3 ± 3.8
	Di.	12.3 ± 8.5	6.0 ± 6.0	26.3 ± 16.6
	F.D.	15.3 ± 5.5	18.3 ± 11.8	8.7 ± 6.2
	L.D.	20.7 ± 5.8	21.7 ± 6.8	9.7 ± 1.8
	No.	5.7 ± 2.3	2.3 ± 1.2	4.0 ± 2.0
	Sh.	1.7 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
OSPIL	Bi.	36.3 ± 25.2	2.7 ± 1.7	113.0 ± 60.9
	Di.	13.7 ± 6.7	7.7 ± 4.7	5.0 ± 2.5
	F.D.	20.3 ± 9.3	14.7 ± 2.9	24.7 ± 6.9
	L.D.	13.3 ± 2.0	17.3 ± 4.3	27.7 ± 12.4
	No.	0.7 ± 0.3	3.7 ± 2.7	0.0 ± 0.0
	Sh.	0.3 ± 0.3	0.7 ± 0.3	1.0 ± 0.6
HYBRID	Bi.	137.7 ± 53.0	53.0 ± 34.5	81.0 ± 20.0
	Di.	14.0 ± 7.1	42.7 ± 20.9	27.7 ± 27.7
	F.D.	40.0 ± 7.1	35.3 ± 1.4	29.3 ± 7.4
	L.D.	23.3 ± 3.0	30.3 ± 7.4	18.7 ± 5.2
	No.	3.7 ± 2.0	6.7 ± 1.2	1.3 ± 1.3
	Sh.	3.3 ± 2.8	0.7 ± 0.3	0.0 ± 0.0

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

Table I.4 Mean (± SE) number of displays performed by each type of female in front of the three male types (irrespective of other male present), during Open-choice experiments (mean across nine tests).

Female type	Beh.*	Male type		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	17.1 ± 3.5	5.3 ± 1.0	29.0 ± 13.3
	Di.	18.7 ± 10.5	10.6 ± 3.7	13.7 ± 6.3
	F.D.	6.4 ± 2.8	7.0 ± 4.5	6.4 ± 2.6
	L.D.	14.8 ± 2.7	12.0 ± 3.7	12.8 ± 2.9
	No.	4.7 ± 1.4	1.7 ± 0.9	5.2 ± 1.3
	Sh.	2.6 ± 0.9	0.7 ± 0.3	1.0 ± 0.5
OSPIL	Bi.	18.0 ± 8.9	6.8 ± 2.6	50.3 ± 24.2
	Di.	13.9 ± 3.6	11.1 ± 4.1	10.8 ± 2.9
	F.D.	11.1 ± 3.9	11.2 ± 2.2	16.0 ± 3.1
	L.D.	9.1 ± 2.5	14.8 ± 2.7	17.6 ± 4.6
	No.	0.6 ± 0.3	2.3 ± 1.0	2.0 ± 0.9
	Sh.	0.2 ± 0.1	1.3 ± 0.8	1.1 ± 0.4
HYBRID	Bi.	60.1 ± 25.6	23.6 ± 37.5	52.2 ± 14.9
	Di.	26.1 ± 8.4	36.0 ± 8.3	36.0 ± 12.1
	F.D.	13.3 ± 7.0	13.7 ± 5.5	11.7 ± 5.0
	L.D.	10.8 ± 3.3	14.0 ± 4.9	15.0 ± 3.7
	No.	1.4 ± 0.8	2.6 ± 1.1	0.8 ± 0.5
	Sh.	2.1 ± 1.0	0.7 ± 0.4	2.0 ± 1.1

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

Table I.5 Mann-Whitney U-test on the number of displays performed by females, for behaviours recorded during Forced-choice experiments, when the two males presented consecutively to females were of the same type (27 experiments) compared to when they were of different types (54 experiments).

Beh.	Two males shown to female		z	*P*
	same type	different type		
Bi.	30.8 ± 8.8	27.2 ± 5.6	-0.511	0.609
Di.	14.6 ± 4.5	9.4 ± 1.9	-0.199	0.842
F.D.	21.0 ± 4.5	4.6 ± 0.6	-6.406	<0.001
L.D.	18.0 ± 1.6	8.7 ± 1.0	-4.624	<0.001
No.	3.3 ± 0.7	1.3 ± 0.3	-2.617	0.009
Sh.	1.2 ± 0.5	2.0 ± 0.5	-1.232	0.218

Beh., see Table I.4 for description of behaviours.

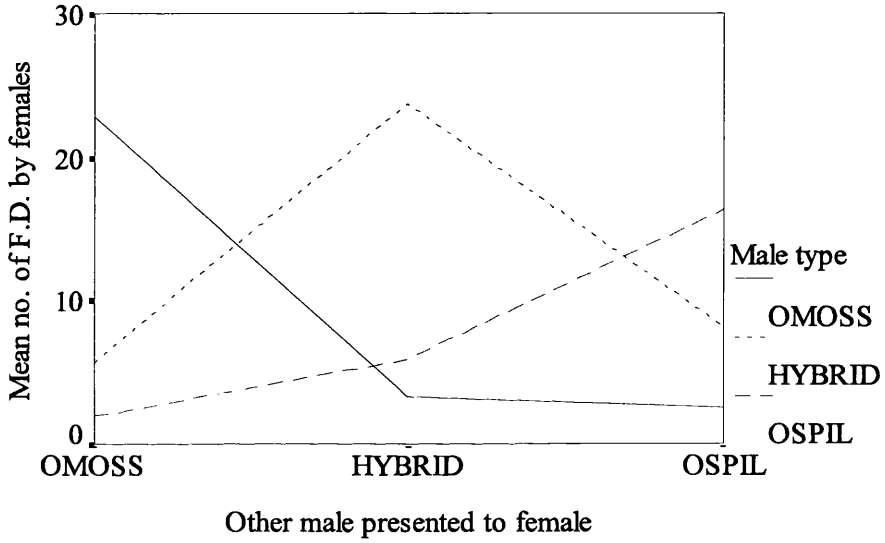

Figure I.1 Mean number of frontal displays performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid females in front of each pair of males presented consecutively during the 15 minute Forced-choice experiments. See Table I.2 for standard error of means.

Table I.6 Mean (± SE) number of displays performed by females, to each type of male, with the three other male types present, during Open-choice experiments (mean across nine tests).

Male type	Beh.	Other male present		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	63.8 ± 25.3	15.0 ± 5.8	16.4 ± 5.7
	Di.	13.3 ± 3.8	18.4 ± 7.1	26.9 ± 11.3
	F.D.	25.2 ± 5.3	1.6 ± 0.8	4.1 ± 2.1
	L.D.	19.1 ± 2.5	5.8 ± 1.9	9.8 ± 2.3
	No.	3.3 ± 1.1	0.9 ± 0.4	2.4 ± 1.4
	Sh.	1.8 ± 1.0	2.3 ± 1.0	0.8 ± 0.4
OSPIL	Bi.	7.3 ± 2.5	20.4 ± 12.9	7.9 ± 1.9
	Di.	19.4 ± 6.8	18.8 ± 8.8	19.4 ± 5.4
	F.D.	4.0 ± 2.5	22.8 ± 4.8	5.1 ± 1.0
	L.D.	7.1 ± 3.1	23.1 ± 3.7	10.6 ± 2.0
	No.	0.7 ± 0.7	4.2 ± 1.1	1.7 ± 0.9
	Sh.	2.0 ± 0.8	0.4 ± 0.2	0.2 ± 0.2
HYBRID	Bi.	21.8 ± 12.7	43.0 ± 12.0	66.8 ± 24.4
	Di.	26.4 ± 10.9	14.3 ± 3.4	19.7 ± 10.0
	F.D.	4.6 ± 2.2	8.7 ± 1.7	20.9 ± 4.7
	L.D.	11.2 ± 3.2	15.4 ± 2.9	18.7 ± 4.7
	No.	4.0 ± 1.5	2.2 ± 0.7	1.8 ± 0.9
	Sh.	2.2 ± 1.1	1.6 ± 0.6	0.3 ± 0.2

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* SPIL, *O. spilurus.*

Table I.7 Mean (± SE) number of displays performed by males, for six behaviours recorded during Forced-choice experiments (three replicates of each combination of female type and male type).

Female	Beh.*	Male type		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	6.6 ± 2.1	19.4 ± 7.2	71.7 ± 26.9
	Di.	16.4 ± 5.7	12.7 ± 5.0	12.2 ± 5.7
	F.D.	1.7 ± 0.7	7.7 ± 2.4	11.8 ± 2.7
	L.D.	13.7 ± 2.2	9.8 ± 2.1	13.8 ± 2.4
	No.	24.1 ± 7.8	10.8 ± 5.3	13.6 ± 6.9
	Sh.	6.8 ± 1.1	4.8 ± 2.4	3.4 ± 2.0
OSPIL	Bi.	6.2 ± 3.6	10.0 ± 6.0	48.6 ± 19.6
	Di.	8.6 ± 3.5	11.9 ± 6.8	25.1 ± 6.1
	F.D.	1.3 ± 0.6	5.1 ± 1.4	6.6 ± 2.1
	L.D.	6.3 ± 1.6	10.8 ± 1.7	12.4 ± 3.2
	No.	11.8 ± 2.8	17.6 ± 4.1	20.3 ± 10.6
	Sh.	4.0 ± 1.2	8.8 ± 1.2	5.2 ± 1.4
HYBRID	Bi.	26.7 ± 14.9	21.9 ± 12.4	49.0 ± 26.8
	Di.	16.6 ± 6.0	9.0 ± 4.6	22.6 ± 5.7
	F.D.	2.3 ± 1.1	5.6 ± 1.4	5.8 ± 3.0
	L.D.	10.3 ± 3.6	7.9 ± 1.8	10.9 ± 3.5
	No.	15.1 ± 7.3	25.3 ± 6.7	21.1 ± 6.4
	Sh.	3.6 ±1.1	11.7 ± 3.1	4.8 ± 1.9

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

Table I.8 Mean (± SE) number of displays performed by males, for six behaviours recorded during Open-choice experiments, when both the males presented to the female are of the same type (three replicates of each).

Female	Beh.*	Both male types presented to female		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	16.3 ± 6.9	22.3 ± 11.0	21.0 ± 9.6
	Di.	11.3 ± 5.7	7.0 ± 5.6	10.7 ± 7.9
	F.D.	6.3 ± 2.8	11.7 ± 4.6	13.7 ± 11.3
	L.D.	14.7 ± 4.9	12.3 ± 1.3	14.0 ± 6.6
	No.	27.7 ± 15.1	20.0 ± 14.0	9.0 ± 2.5
	Sh.	1.7 ± 0.9	7.3 ± 5.9	1.7 ± 1.2
OSPIL	Bi.	18.7 ± 10.7	20.0 ± 12.2	134.3 ± 66.3
	Di.	14.7 ± 7.2	5.7 ± 2.6	4.0 ± 2.6
	F.D.	1.3 ± 0.9	16.7 ± 3.5	12.3 ± 5.5
	L.D.	3.0 ± 2.5	11.0 ± 4.3	13.0 ± 9.0
	No.	17.3 ± 5.9	9.3 ± 3.9	12.7 ± 10.3
	Sh.	0.3 ± 0.3	2.3 ± 0.9	4.0 ± 3.0
HYBRID	Bi.	79.0 ± 32.4	97.7 ± 87.3	106.7 ± 52.3
	Di.	5.3 ± 3.9	4.3 ± 1.8	16.7 ± 13.8
	F.D.	0.0 ± 0.0	17.7 ± 5.4	6.7 ± 3.8
	L.D.	5.0 ± 5.0	12.3 ± 0.3	4.0 ± 2.3
	No.	50.0 ± 1.1	41.3 ± 15.8	25.3 ± 9.4
	Sh.	2.3 ± 0.9	9.3 ± 4.8	4.0 ± 1.1

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

Table I.9 Mean (± SE) number of displays performed by each type of male in front of the three types of female (irrespective of the other male present), for six behaviours recorded during Open-choice experiments (mean across nine tests).

Female type	Beh.*	Male type		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	10.3 ± 3.5	25.4 ± 7.3	91.4 ± 34.3
	Di.	16.3 ± 5.2	5.3 ± 2.4	21.0 ± 12.6
	F.D.	4.9 ± 1.5	9.3 ± 2.6	9.8 ± 3.6
	L.D.	15.3 ± 2.0	10.8 ± 2.1	14.7 ± 2.1
	No.	20.3 ± 6.2	12.9 ± 6.4	7.0 ± 4.3
	Sh.	5.4 ± 1.5	5.0 ± 2.3	2.1 ± 1.1
OSPIL	Bi.	8.4 ± 4.4	47.2 ± 16.2	108.3 ± 25.4
	Di.	9.2 ± 3.2	6.4 ± 2.3	13.3 ± 5.2
	F.D.	1.3 ± 0.5	13.0 ± 2.7	9.4 ± 3.1
	L.D.	7.0 ± 2.2	15.0 ± 2.5	13.2 ± 4.4
	No.	11.9 ± 2.8	13.0 ± 2.6	8.7 ± 3.3
	Sh.	4.0 ± 2.3	4.7 ± 1.5	3.4 ± 1.4
HYBRID	Bi.	30.6 ± 15.4	47.7 ± 29.7	60.6 ± 27.6
	Di.	11.8 ± 4.7	3.9 ± 1.0	30.3 ± 9.0
	F.D.	1.6 ± 0.9	9.1 ± 3.1	9.4 ± 3.7
	L.D.	5.9 ± 1.9	8.3 ± 1.8	9.4 ± 2.8
	No.	18.3 ± 8.0	25.2 ± 6.8	15.4 ± 5.3
	Sh.	3.9 ± 1.1	5.9 ± 2.3	2.4 ± 0.6

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

Table I.10 Mann-Whitney U-test (z-value and probability) on the number of displays performed by males, for behaviours recorded during Forced-choice experiments, when the two males presented consecutively to females were of the same type (27 experiments) compared to when they were of a different type (54 experiments).

Beh.	Two males shown to female		z	*P*
	same type	different type		
Bi.	25.3 ± 7.8	30.7 ± 7.5	−0.660	0.560
Di.	16.3 ± 3.4	14.3 ± 2.2	−0.242	0.809
F.D.	5.9 ± 1.3	5.0 ± 0.8	−0.010	0.992
L.D.	8.6 ± 1.1	11.7 ± 1.1	−1.455	0.146
No.	31.9 ± 4.9	10.7 ± 1.6	−4.046	<0.001
Sh.	6.7 ± 1.3	5.4 ± 0.7	−0.563	0.573

Beh., see Table I.9 for description of behaviours.

Table I.11 Mean (± SE) number of displays performed by the three male types, in front of all females, with each type of other male present, during Open-choice experiments (mean across nine tests).

Male type	Beh.	Other male present		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	38.0 ± 14.4	4.8 ± 2.4	6.5 ± 10.3
	Di.	10.4 ± 3.2	11.8 ± 4.6	15.1 ± 5.5
	F.D.	2.6 ± 1.3	3.7 ± 1.5	1.6 ± 0.4
	L.D.	7.6 ± 2.8	9.2 ± 2.4	11.4 ± 2.1
	No.	31.7 ± 6.7	5.7 ± 2.5	13.2 ± 4.4
	Sh.	1.4 ± 0.5	4.7 ± 1.4	7.2 ± 2.1
OSPIL	Bi.	24.5 ± 12.4	46.7 ± 28.6	49.1 ± 14.7
	Di.	2.8 ± 1.2	5.7 ± 1.9	7.2 ± 2.5
	F.D.	5.9 ± 2.7	15.3 ± 2.5	10.2 ± 2.5
	L.D.	9.4 ± 2.5	11.9 ± 1.3	12.8 ± 2.9
	No.	8.2 ± 3.3	23.6 ± 7.8	19.3 ± 4.4
	Sh.	6.2 ± 2.0	6.3 ± 2.4	3.0 ± 1.3
HYBRID	Bi.	86.0 ± 29.5	87.0 ± 31.0	87.3 ± 29.9
	Di.	30.6 ± 8.9	23.7 ± 12.5	10.4 ± 5.0
	F.D.	13.3 ± 3.7	4.4 ± 1.3	10.9 ± 3.9
	L.D.	15.4 ± 4.0	11.6 ± 2.4	10.3 ± 3.7
	No.	6.4 ± 1.6	9.0 ± 3.9	15.7 ± 4.8
	Sh.	3.9 ± 1.3	0.9 ± 0.4	3.2 ± 1.1

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* OSPIL, *O. spilurus.*

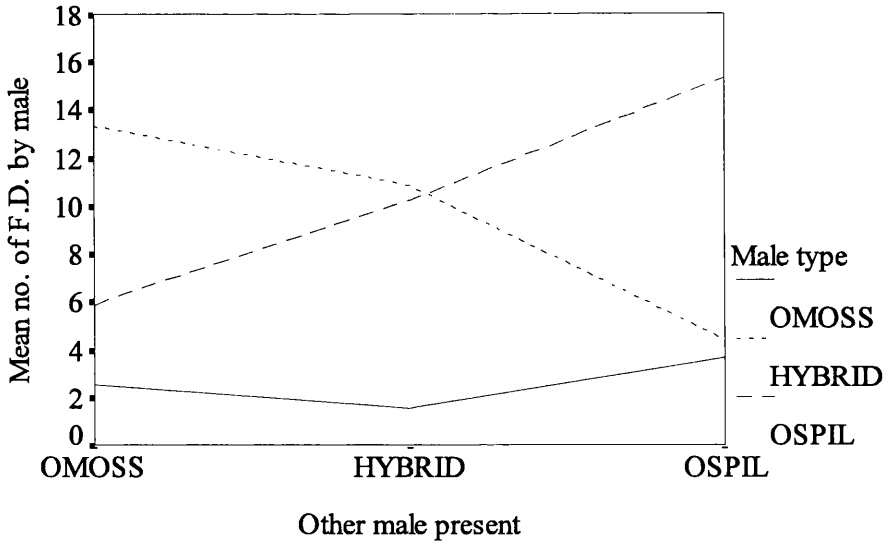

Figure I.2 Mean number of frontal displays performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid males in front of all females, with each type of other male present, during the 30 minute Open-choice experiments. See Table I.11 for standard error of means.

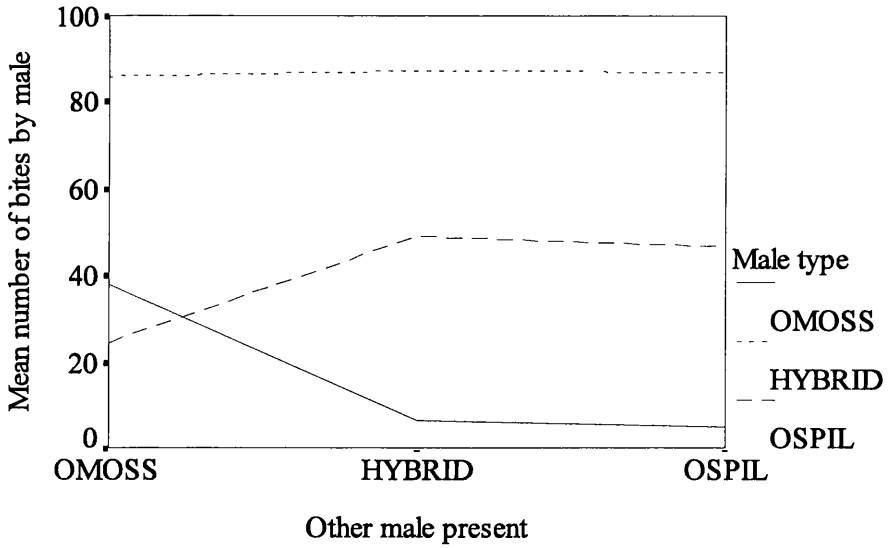

Figure I.3 Mean number of bites performed by *O. mossambicus* (OMOSS), *O. spilurus* (OSPIL) and hybrid males in front of all females, with each type of other male present, during the 30 minute Open-choice experiments. See Table I.11 for standard error of means.

Table I.12 Mean (± SE) number of displays performed by males in front of the three types of female, with each type of other male present, for six behaviours recorded during the Open-choice experiments (mean across nine tests).

Female	Beh.	Other male present		
		OMOSS	OSPIL	HYBRID
OMOSS	Bi.	54.7 ± 26.8	53.0 ± 29.0	19.6 ± 6.9
	Di.	5.6 ± 2.3	23.1 ± 12.5	14.0 ± 5.3
	F.D.	6.0 ± 1.5	9.3 ± 1.7	8.7 ± 4.2
	L.D.	11.2 ± 2.8	15.0 ± 1.5	14.6 ± 2.4
	No.	12.4 ± 5.9	10.2 ± 5.2	17.6 ± 5.2
	Sh.	3.1 ± 1.2	5.3 ± 2.2	4.1 ± 1.5
OSPIL	Bi.	65.2 ± 20.9	28.1 ± 13.2	70.7 ± 28.6
	Di.	15.3 ± 5.1	7.7 ± 3.0	6.0 ± 2.2
	F.D.	9.3 ± 3.3	6.0 ± 2.9	8.4 ± 2.5
	L.D.	13.8 ± 3.9	7.6 ± 1.9	13.9 ± 3.6
	No.	13.8 ± 2.8	6.1 ± 1.7	13.7 ± 3.4
	Sh.	5.1 ± 1.7	1.6 ± 0.6	5.4 ± 2.2
HYBRID	Bi.	28.7 ± 15.7	57.3 ± 33.7	52.8 ± 22.2
	Di.	22.9 ± 9.6	10.3 ± 4.3	12.8 ± 5.3
	F.D.	6.4 ± 4.1	8.1 ± 2.9	5.6 ± 1.9
	L.D.	7.4 ± 2.8	10.1 ± 2.0	6.1 ± 1.6
	No.	20.1 ± 7.8	21.9 ± 7.7	17.0 ± 4.9
	Sh.	3.3 ± 1.6	5.0 ± 1.9	3.9 ± 1.1

* Behaviours: Bi., biting; Di., digging; F.D., frontal display; L.D., lateral display; No., nodding; Sh., shaking. OMOSS, *O. mossambicus;* OSPIL, *O. spilurus;* O.m.p; Other male present..

APPENDIX II: ALLOZYME ELECTROPHORESIS

Table II.1 Enzyme systems investigated, enzyme number, tissue source and buffer system used. DDH, G6PDH and HK enzyme systems produced insufficient resolution and/or activity during initial screening, so were therefore not used any further

Enzyme	EC No.	Tissue source *	Electrode buffer †
Adenosine deaminase (ADA)	2.6.1.1	M, L	TBE
Alanine aminotransferase (ALAT)	2.6.1.2	L	TBE
Creatine kinase (CK)	2.7.3.2	M	TC
Dihydrolipoamide dehydrogenase (DDH)	1.8.1.4	M	TBE
Esterase (EST)	3.1.1.-	M	TC
Glucose-6-phosphate dehydrogenase (G6PDH)	1.1.1.49	L	TCB
Glucose-6-phoshate isomerase (PGI)	5.3.1.9	M, L	TC
Hexokinase (HK)	2.7.1.1	M	TBE
L-Iditol dehydrogenase (IDDH)	1.1.1.14	L	TC
Isocitrate dehydrogenase (IDH)	1.1.1.42	M, L	TC
L-Lactate dehydrogenase (LDH)	1.1.1.27	M, L	TBE
Malate dehydrogenase (MDH)	1.1.1.37	M, L	TBE
Phosphogluconate dehydrogenase (PGDH)	1.1.1.44	M, L	TC
Phosphoglucomutase (PGM)	5.4.2.2	M	TC
Superoxide dismutase (SOD)	1.15.1.1	M, L	TBE

* M, muscle; L, liver. † Electrode buffers details shown in Table II.2

Table II.2 Buffer systems used for allozyme electrophoresis. Adapted from McAndrew & Majumdar (1983) and Sodsuk & McAndrew (1991). All systems were run at 200 Volts.

	Electrode (g/l)	Gel (g/l)	Run time
TBE Tris-Borate-EDTA (pH 8.5)	60.57 g Tris 5.99 g EDTA 15.00 g Boric acid	Dilute 1:10	4 h
TC Tris-citrate (pH 8.0)	30.29 g Tris 11.98 g Citric acid (monohydrate)	Dilute 1:25	4 h
TCB Tris-citrate/borate (pH 8.6)	18.60 g Boric acid 4.20 g Lithium hydroxide	9.20 g Tris 1.05 g Citric acid 53.0 ml Electrode buffer	5 h

Table II.3 Recipes used for staining for enzyme loci, modified from Aebersold *et al.* (1987), Ferguson (1985) and Murphy *et al.*(1996).

Enzyme*	Ingredients†	Stain buffer	Linking enzyme/s
ADA	15 mg Adenosine	15 ml 0.1M Phosphate buffer, pH 7.0	25 μl Nucleoside phosphorylase 20 μl Xanthine oxidase
ALAT	1 ml NAD+ (10 mg /ml) 7 mg ADP 0.5 mg Pyridoxal-5-phosphate 20 mg L-Alanine	15 ml 0.1M Phosphate, 0.1% Ketoglutaric acid, pH 8.5	50 μl (100 units) Glutamate dehydrogenase (suspended in 50% glycerol)
CK	25 mg Phosphocreatine 25 mg ADP 100 mg α-D-glucose 1 ml $MgCl_2$ (0.2 g / 20ml) 1 ml NADP(10mg /ml)	15 ml 0.2M Tris-HCL buffer, pH 8.0	30 μl Hexokinase 10 μl Glucose-6-phosphate dehydrogenase (G6PDH)
EST	10 mg 4-methylumbelliferyl acetone (dissolved in 2 ml acetone)	30 ml 0.1M Phosphate buffer, pH 7.0	
GPI	20 mg Fructose-6-phosphate 1 ml $MgCl_2$ (0.2g / 10 ml) 1 ml NADP (10mg / ml)	15 ml 0.1M Tris-HCL buffer, pH 8.0	10 μl G6PDH
IDH	1 ml NADP (10mg / ml) 50 mg Na_3-Isocitrate 1 ml $MgCl_2$ (0.2g / 10 ml)	15 ml 0.1M Tris-HCL buffer, pH 7.4	
IDDH	1 ml NAD+ (10 mg /ml) 2 ml 50% Sorbital	13 ml 0.2M Tris-HCL buffer, pH 8.5	
LDH	4 ml Lithium lactate, pH 8.0 1 ml NAD+ (10 mg /ml)	8 ml 0.2M Tris-HCL buffer, pH 8.0	
MDH	1 ml NAD+ (10 mg /ml) 2 ml 1.0M Malic acid / NaOH, pH 7.0	13 ml 0.2M Tris-HCL buffer, pH 8.0	
PGDH	1 ml $MgCl_2$ (0.2 g / 20ml) 1 ml NADP(10mg /ml) 20 mg 6-Phosphogluconic acid	15 ml 0.2M Tris-HCL buffer, pH 8.0	
PGM	1 ml $MgCl_2$ (0.2 g / 20ml) 50 mg α-D-glucose-1-phosphate 1 ml NADP (10 mg /ml)	12 ml 0.2M Tris-HCL buffer, pH 8.0	10 ml G6PDH

† All stains, except EST, also contained 1 ml 10 mg / ml MTT (Tetrazolium salt), 1 ml 10 mg / ml PMS (Phenazine methosulfate) and 15 ml 2% agar solution.
The stain for ALAT, and sometimes for IDH or IDDH, also stained for SOD.
* Enzyme details and full name shown in Table II.1.

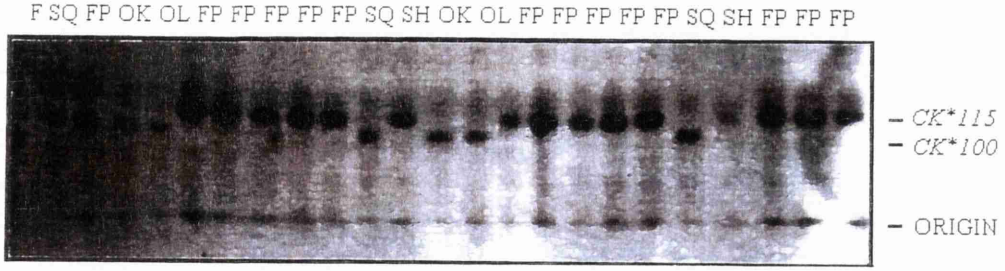

Figure II.1 Creatine kinase (CK)

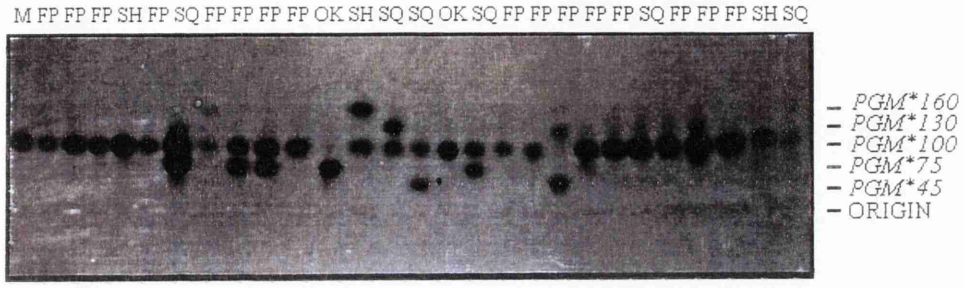

Figure II.2 Phosphoglucomutase (PGM)

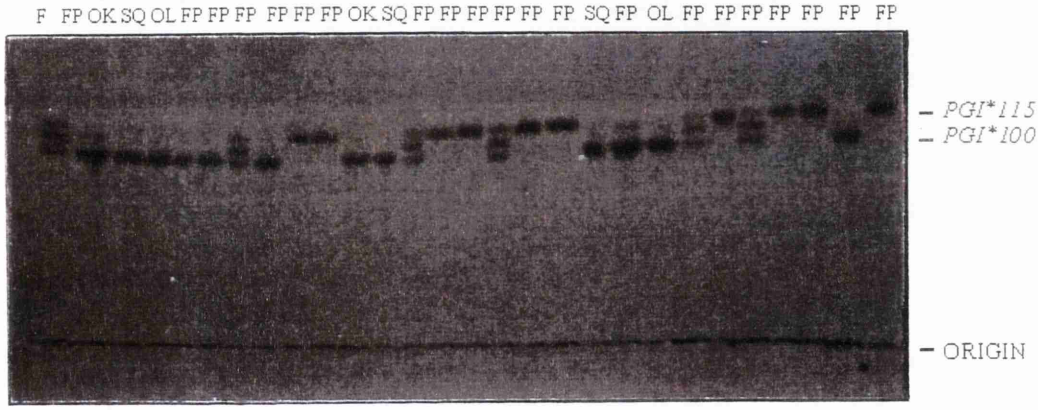

Figure II.3 Glucose-6-phosphate isomerase (PGI)

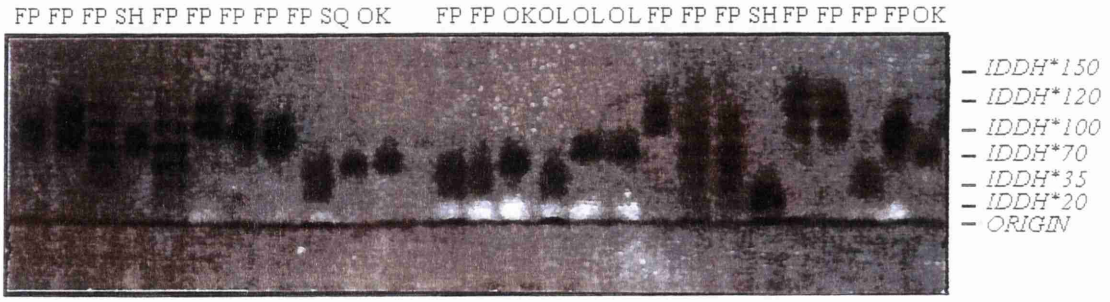

Figure II.4 L-Iditol dehydrogenase (IDDH)

Figure II.1-II.4 Examples of allozyme electrophoresis gels produced in the study of Lake Malawi *Oreochromis*species and five farm pond populations (Nb not all the alleles found at each locus are shown). F, Ferritin marker; M, *O. mossambicus*; FP, Farm pond fish (putative hybrids); OK, *O. karongae*; OL, *O. lidole*; SQ, *O. squamipinnis*; SH; *O. shiranus*.

Table II.4 Allelic variation of Lake Malawi *Oreochromis* species and five farm pond populations sampled in Malawi, examined at 13 enzyme loci.

		Species*				Pond population*				
Locus	Allele	OSHI	OSQU	OKAR	OLID	DMSH	DMOK	DWSE	DWST	MZKH
		n=20	n=23	n=21	n=18	n=10	n=9	n=30	n=47	n=10
*ADA**	118 †	0.000	0.065	0.024	0.028	0.050	0.056	0.033	0.191	0.000
	108	0.000	0.174	0.119	0.056	0.200	0.111	0.000	0.160	0.100
	104	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	100	0.000	0.130	0.143	0.028	0.000	0.278	0.000	0.128	0.200
	93	0.000	0.609	0.666	0.805	0.000	0.555	0.017	0.245	0.650
	80 †	0.000	0.000	0.048	0.083	0.150	0.000	0.100	0.149	0.050
	66	0.550	0.000	0.000	0.000	0.300	0.000	0.417	0.117	0.000
	60 ◊	0.450	0.000	0.000	0.000	0.300	0.000	0.433	0.011	0.000
*ALAT**	100	1.000	0.000	0.000	0.000	1.000	0.222	1.000	0.213	0.250
	87	0.000	1.000	1.000	1.000	0.000	0.778	0.000	0.574	0.750
	60 #	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.213	0.000
*CK**	115	1.000	0.000	0.000	0.000	1.000	0.056	1.000	0.649	0.000
	100	0.000	1.000	1.000	1.000	0.000	0.888	0.000	0.351	1.000
	67 #	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.000
*EST**	108 #	0.000	0.000	0.000	0.000	0.150	0.056	0.000	0.074	0.000
	100	1.000	0.913	1.000	1.000	0.700	0.888	0.867	0.681	1.000
	93	0.000	0.065	0.000	0.000	0.100	0.056	0.083	0.160	0.000
	77 †	0.000	0.022	0.000	0.000	0.050	0.000	0.050	0.085	0.000
*IDDH**	150 #	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.011	0.000
	120 †	0.000	0.000	0.048	0.055	0.000	0.000	0.217	0.106	0.000
	100	0.725	0.000	0.000	0.000	0.900	0.000	0.283	0.340	0.000
	70	0.000	1.000	0.928	0.917	0.100	1.000	0.150	0.457	1.000
	35 †	0.000	0.000	0.024	0.028	0.000	0.000	0.117	0.021	0.000
	20 ◊	0.275	0.000	0.000	0.000	0.000	0.000	0.150	0.064	0.000
*LDH-1**	100	1.000	0.891	0.690	0.444	1.000	0.611	1.000	0.979	0.900
	-100	0.000	0.109	0.310	0.556	0.000	0.389	0.000	0.021	0.100
*LDH-2**	100	1.000	1.000	1.000	1.000	0.850	1.000	0.967	0.266	1.000
	180	0.000	0.000	0.000	0.000	0.150	0.000	0.033	0.734	0.000
*PGDH**	116 ◊	0.100	0.000	0.000	0.111	0.200	0.222	0.183	0.043	0.300
	100	0.900	1.000	1.000	0.889	0.800	0.778	0.817	0.957	0.700
*PGI**	115 #	0.000	0.000	0.000	0.000	0.150	0.000	0.000	0.691	0.000
	100	1.000	1.000	1.000	1.000	0.850	1.000	1.000	0.287	1.000
	89 #	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.000
*PGM**	160 ◊	0.125	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000
	130 †	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.021	0.000
	100	0.875	0.913	0.905	1.000	1.000	1.000	0.950	0.947	0.800
	75	0.000	0.043	0.095	0.000	0.000	0.000	0.000	0.021	0.200
	45 †	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.011	0.000
*SOD**	100	0.575	0.239	0.071	0.278	0.150	0.111	0.733	0.138	0.100
	25	0.425	0.761	0.929	0.722	0.850	0.889	0.267	0.862	0.900

* species / populations abbreviations as in Chapter 3.

Based on Sodsuk *et al.* (1995): †, alleles not previously observed in any chambo species; ◊, alleles not previously observed in *O. shiranus;* #, alleles unique to farms and not previously observed in *O. shiranus* or chambo species.

Table II.5 Frequency of each hybrid genotype, based on four diagnostic loci, found in the five farm ponds sampled in Malawi.

Genotype †			No. of each genotype found in farm ponds*				
no. of loci *SC*	no. of loci *SS*	no. of loci *CC*	DMSH n=7/10	DMOK n=2/9	DWSE n=22/30	DWST n=43/47	MZKH n=3/10
4	0	0	-	-	-	-	-
3	1	0	-	-	-	-	-
3	0	1	0	0	0	3	0
2	2	0	1	0	1	1	0
2	0	2	0	0	0	2	0
2	1	1	0	0	0	2	0
1	3	0	3	0	5	0	0
1	0	3	0	0	0	7	1
1	2	1	1	0	0	4	0
1	1	2	0	1	0	9	0
0	3	1	2	0	14	0	0
0	1	3	0	1	0	9	2
0	2	2	0	0	2	6	0

†Genotype: number of loci homozygous for *O. shiranus* (*SS*), homozygous for chambo (*CC*) and heterozygous (*SC*)
* DMSH, *O. shiranus* pond Domasi; DMOK, *O. karongae* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storage pond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu.
- No individuals found in any pond with these genotypes; n = total number of hybrids / total number of fish.

Table II.6 Genetic differentiation between all pairs of populations sampled. Loci showing significant differentiation in allelic composition (above the diagonal) and significance over all loci in each pairwise comparison (below the diagonal).

	OSHI	OSQU	OKAR	OLID	DMSH	DMOK	DWSE	DWST	MZKH
OSHI		*ADA *** ALAT *** CK *** IDDH *** (PGDH *) PGM * SOD ***	*ADA *** ALAT *** CK *** IDDH *** LDH-1 *** (PGDH *) PGM ** SOD ****	*ADA *** ALAT *** CK *** IDDH *** LDH-1 *** SOD **	*ADA *** EST ** IDDH ** (LDH-2 *) (PGI *) SOD ***	*ADA *** ALAT *** CK *** IDDH *** LDH-1 *** SOD ***	*(EST *) IDDH ****	*ADA *** ALAT *** CK *** EST ** IDDH *** LDH-2 *** PGI ** PGM **	*ADA *** ALAT*** CK *** IDDH *** PGM * SOD ****
OSQU	*H.S*		*(LDH-1 *) SOD **	*ADA * LDH-1 *** (PGDH *)*	*ADA *** ALAT *** CK *** (EST *) IDDH *** (LDH-2 *) PGDH * (PGI *)*	*ALAT * (LDH-1 *) PGDH ***	*ADA *** ALAT *** CK *** IDDH *** LDH-1 * PGDH ** (PGM *) SOD ****	*ADA *** ALAT*** CK *** (EST *) IDDH * (LDH-1 *) LDH-2 *** PGI ****	*ALAT ** PGDH ***
OKAR	*H.S*	*H.S*		*(LDH-1 *) (PGDH *) (SOD *)*	*ADA*** ALAT *** CK *** EST *** IDDH *** LDH-1 * (LDH-2 *) PGDH * (PGI *)*	*(ALAT *) (PGDH *)*	*ADA *** ALAT*** CK *** (EST*) IDDH*** LDH-1 *** PGDH ** PGM * SOD ****	*ADA *** ALAT *** CK *** EST *** IDDH *** LDH-1 *** LDH-2 *** PGI ****	*ALAT** PGDH ****
OLID	*H.S*	***	*		*ADA *** ALAT *** CK *** EST ** IDDH *** LDH-1 *** (LDH-2 *) (PGI *)*	*(ADA *) (ALAT *)*	*ADA *** ALAT *** CK *** IDDH *** LDH-1 *** SOD ****	*ADA *** ALAT *** CK *** EST *** IDDH *** LDH-1 *** LDH-2 *** PGI ****	*ALAT * LDH-1 ** (PGM *)*
DMSH	***	*H.S*	*H.S*	*H.S*		*ADA *** ALAT *** CK *** IDDH *** LDH-1 ***	*(ADA *) (EST *) IDDH *** (PGI *) SOD ****	*ADA *** ALAT *** CK *** IDDH *** LDH-2 *** (PGDH *) PGI ****	*ADA *** ALAT *** CK *** (EST *) IDDH ****
DMOK	*H.S*	**	*	*	*H.S*		*ADA *** ALAT *** CK *** IDDH *** LDH-1 *** SOD ****	*(ADA *) CK *** IDDH ** LDH-1 *** LDH-2 *** (PGDH *) PGI ****	
DWSE	*H.S*	*H.S*	*H.S*	*H.S*	*H.S*	*H.S*		*ADA *** ALAT *** CK *** (EST *) IDDH *** LDH-2 *** PGDH ** PGI *** SOD ****	*ADA *** ALAT *** CK *** IDDH *** PGM ** SOD ****
DWST	*H.S*	*H.S*	*H.S*	*H.S*	*H.S*	*H.S*	*H.S*		*ADA * (ALAT *) CK *** (EST *) IDDH *** LDH-2 *** PGDH ** PGI *** (PGM *)*
MZKH	*H.S*	***	**	***	*H.S*	*n.s*	*H.S*	*H.S*	

Species / populations abbreviations as in Chapter 3.
Significant levels: * p<0.05; ** p<0.01; *** p<0.005; *H.S,* Highly significant; *n.s,* Not significant. Results in parenthesis are no longer significant after the sequential Bonferroni test is applied (5% significance level).

Table II.7 Measures of genetic differentiation between pairs of samples. Reynolds *et al.* (1983) genetic distances (above diagonal) and Cavalli-Sforza's chord measure (Cavalli-Sforza & Edwards 1967) (below diagonal).

	OSHI	OSQU	OKAR	OLID	DMSH	DMOK	DWSE	DWST	MZKH
OSHI		0.6782	0.6947	0.6884	0.1729	0.5997	0.0917	0.4411	0.6256
OSQU	0.5957		0.0571	0.1488	0.6217	0.1050	0.6109	0.4034	0.1068
OKAR	0.6207	0.0292		0.0882	0.6327	0.0763	0.6336	0.4176	0.1126
OLID	0.6131	0.0568	0.0289		0.6388	0.0957	0.6232	0.4412	0.1869
DMSH	0.1266	0.5373	0.5617	0.5677		0.5333	0.2128	0.3245	0.5612
DMOK	0.5213	0.0656	0.0663	0.0617	0.4407		0.5353	0.3625	0.0680
DWSE	0.0744	0.5292	0.5469	0.5217	0.1233	0.4562		0.3986	0.5609
DWST	0.4307	0.3099	0.3245	0.3482	0.2420	0.2909	0.3337		0.3830
MZKH	0.5365	0.0676	0.0547	0.0734	0.4747	0.0474	0.4759	0.3210	

OSHI, *O. shiranus*; OSQU, *O. squamipinnis*; OKAR, *O. karongae*; OLID, *O. lidole*; DMSH, *O. shiranus* pond Domasi; DMOK, *O. karongae* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storagepond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu.

Table II.8 Measures of dissimilarity between pairs of samples; Average Manhattan distances (Rohlf 1992).

	OSHI	OSQU	OKAR	OLID	DMSH	DMOK	DWSE	DWST
OSHI								
OSQU	0.2213							
OKAR	0.2377	0.0324						
OLID	0.2320	0.0527	0.0394					
DMSH	0.0895	0.2174	0.2322	0.2375				
DMOK	0.2295	0.0583	0.0521	0.0608	0.2046			
DWSE	0.0558	0.2210	0.2374	0.2297	0.0915	0.2201		
DWST	0.2331	0.1844	0.1964	0.2169	0.1719	0.1902	0.2155	
MZKH	0.2198	0.0513	0.0477	0.0731	0.2116	0.0453	0.2209	0.1958

OSHI, *O. shiranus*; OSQU, *O. squamipinnis*; OKAR, *O. karongae*; OLID, *O. lidole*; DMSH, *O. shiranus* pond Domasi; DMOK, *O. karongae* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storagepond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu.

APPENDIX III: RAPD

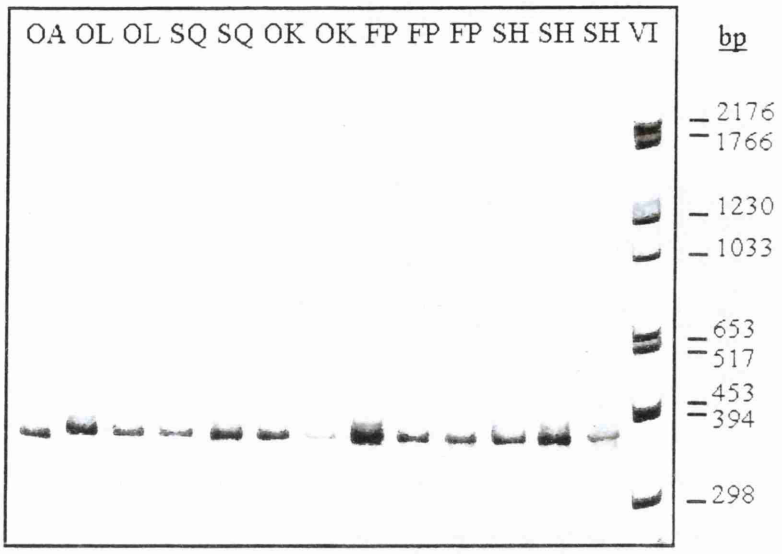

Figure III.1 SCAR products of primer OPB 06 $_{374}$.
SH, *O. shiranus*; FP, Farm pond; OK, *O. karongae*; SQ, *O. squamipinnis*; OL, *O. lidole;* OA, *O. aureus;*VI, Boehringer marker VI. Basepair lengths (bp) illustrated to right of gel.

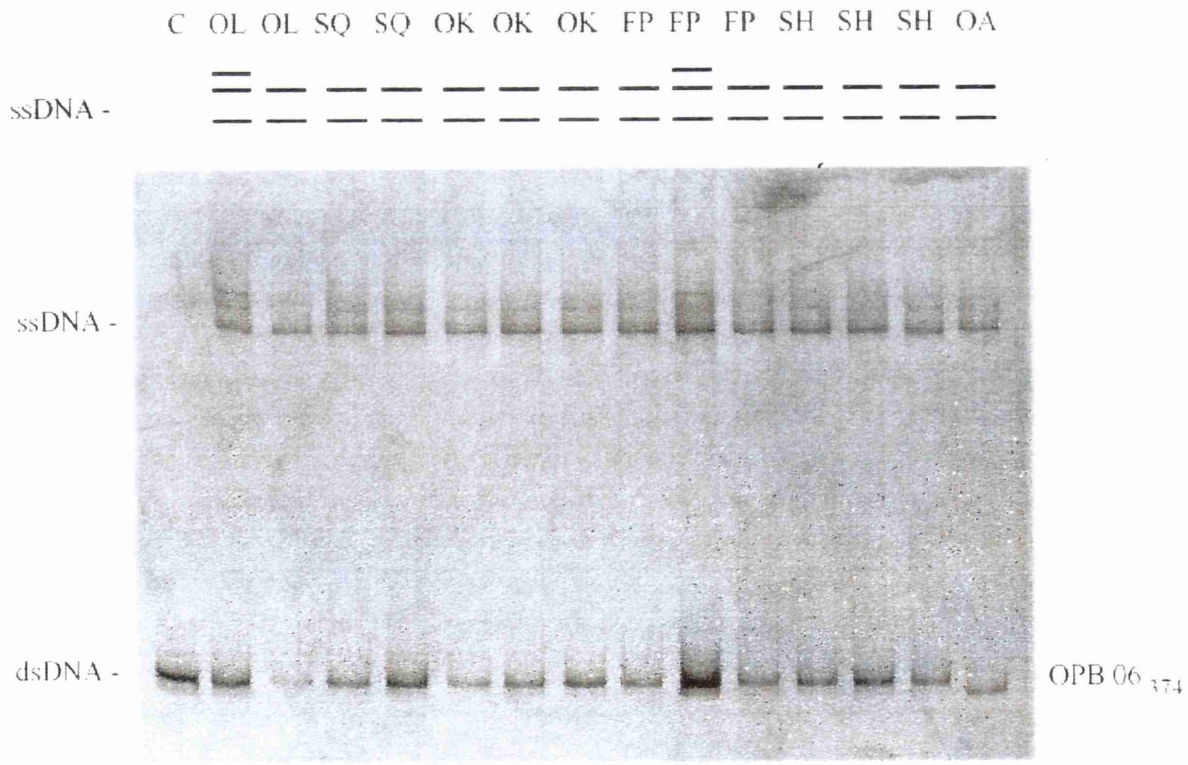

Figure III.2 Gel and interpretation (diagram above gel) of fragments generated by the SSCP analysis of the products of SCAR primer OPB 06 $_{374}$.
C, control; OA, *O. aureus*; SH, *O. shiranus*; FP, farm pond; OK, *O. karongae*; SQ, *O. squamipinnis*; OL, *O. lidole*; ds, double-stranded DNA; ss, single-stranded DNA.

Table III.1 Frequency of chambo and *O. shiranus* diagnostic fragments, based on the analyses with four RAPD primers, of each hybrid found in the seven farm ponds sampled in Malawi.

Combination of diagnostic fragments		Farm pond (abbreviations as in Chapter 4)						
Chambo fragments (out of 5)	*O. shiranus* fragments (out of 4)	DMSH n=15	DMOK n=15	DWSE n=27	DWST n=18	MZKH n=10	MZKB n=18	MZSH n=15
4	3	0	0	0	1	0	0	0
4	2	0	0	0	1	0	0	0
3	4	1	0	1	0	0	0	0
3	3	0	0	0	2	0	0	0
3	1	0	0	0	0	2	1	0
2	3	1	1	1	0	0	0	0
2	2	1	0	0	4	0	0	0
1	4	5	0	9	0	0	0	0
1	3	2	0	3	0	0	0	1
1	2	0	0	1	2	0	0	0
Total		10	1	15	10	2	1	1

Table III.2 Chi-square (Nass, 1959) values for fragments with significant differences ($p<0.05$) in the distribution of their presence and absence in pairwise comparison of the three chambo species, based on their analyses with four RAPD primers.

Pairwise comparison	Fragment	X^2	*df*	*P*
OKAR / OSQU	A08.1330	4.7174	1.0610	0.05
	A08.0970	4.7174	1.0610	0.05
	A08.0815	6.1996	1.3917	0.025
	A08.0806	6.6766	1.1064	0.01
	A08.0680	6.6766	1.1064	0.01
	A08.0630	6.1999	1.3917	0.025
	A08.0625	9.5693	1.0526	0.005
	A08.0480	9.3907	1.0865	0.005
	A10.1630	5.6735	1.1347	0.025
	A10.1545	4.0190	1.1776	0.05
	A10.1430	4.8040	1.0720	0.05
	A10.1400	7.0775	1.0526	0.01
	A10.0825	11.4741	1.0720	0.001
	A10.0692	4.7174	1.0610	0.05
	A10.0575	5.6735	1.1347	0.025
	A10.0565	7.4615	1.1064	0.01
	A10.5300	9.3067	1.1776	0.005
	E15.0930	8.6350	1.0610	0.005
	E15.0605	4.3724	1.0316	0.05
	G17.0864	3.9040	1.2493	0.05
	G17.0726	15.6315	1.0412	0.001
	G17.0549	6.1996	1.3917	0.025
	G17.0418	7.4615	1.1064	0.01
	G17.0410	7.7609	1.0865	0.01
OKAR / OLID	A08.1330	7.4689	1.1457	0.01
	A08.1068	4.1237	1.0488	0.05
	A08.0720	6.0272	0.9642	0.025
	A08.0480	12.4175	1.0488	0.001
	A10.0565	6.0272	0.9642	0.025
	A10.0550	16.5664	0.9642	0.001
	E15.0690	9.8424	1.2111	0.001
	G17.1058	4.4121	1.2284	0.05
	G17.0444	6.0993	1.2316	0.025
OSQU / OLID	A08.0720	4.8559	1.1493	0.05
	A08.0640	5.1962	1.2028	0.025
	A10.1046	4.9971	1.1931	0.05
	A10.0550	6.9515	1.1848	0.01
	E15.0690	9.8601	1.1983	0.001

df, degrees of freedom; *P*, probability values; OKAR, *O. karongae*; OSQU, *O. squamipinnis*; OLID, *O. lidole*.

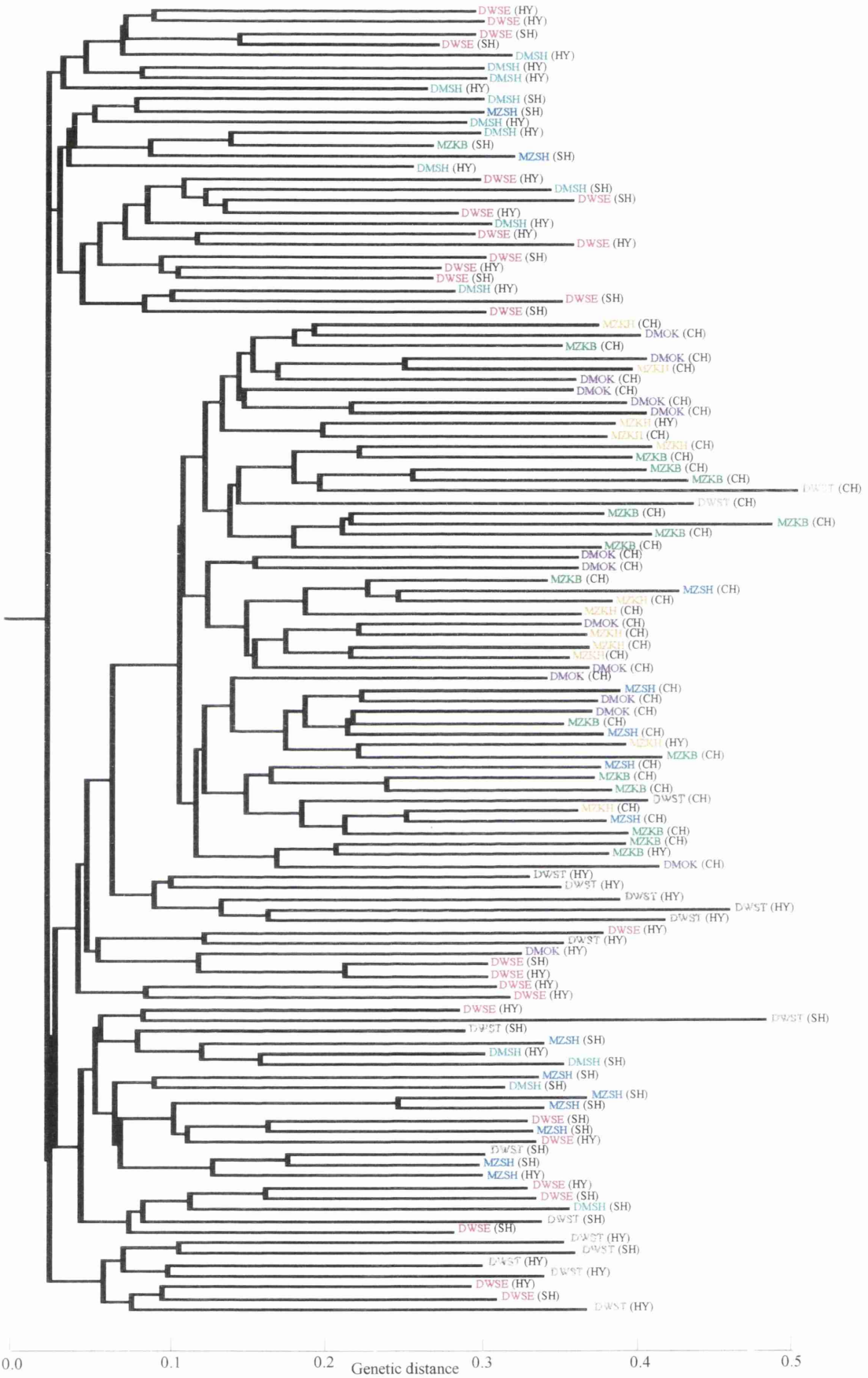

Figure III.3 RAPD-generated neighbour-joining tree of fish from all farm ponds sampled in Malawi (DMOK, Domasi *O. karongae;* DMSH, Domasi *O. shiranus;* DWSE, Dwangwa sewage tank; DWST, Dwangwa storage tank; MZKB, Mzuzu *O. karongae* breeding; MZKH , Mzuzu *O. karongae* holding; MZSH, Mzuzu *O. shiranus*). Identity of fish, based on nine diagnostic RAPD fragments, shown in parentheses (SH, *O. shiranus;* CH, Chambo; HY hybrid).

Table III.3 Mean genetic divergence (Nei & Tajima 1981; DA program REAP, McElroy *et al.* 1991) between all pairs of samples, calculated from the distance matrix of all individuals (based on the analysis of 163 RAPD fragments) generated by RAPDPLOT (Black 1995).

	OSHI n=20	OSQU n=15	OKAR n=21	OLID n=4	DMSH n=15	DMOK n=15	DWSE n=27	DWST n=18	MZKH n=10	MZKB n=18	MZSH n=15	OAUR n=7	OMOS n=7
OSHI													
OSQU	0.1584												
OKAR	0.1492	0.0190											
OLID	0.2228	0.0269	0.0512										
DMSH	0.0066	0.1046	0.0947	0.1770									
DMOK	0.1295	0.0253	0.0102	0.0626	0.0805								
DWSE	0.0140	0.0122	0.1332	0.1846	0.0139	0.1203							
DWST	0.0122	0.0412	0.0670	0.0997	0.0287	0.0577	0.0378						
MZKH	0.1506	0.0455	0.0040	0.0938	0.0945	0.0090	0.1520	0.0767					
MZKB	0.1423	0.0548	0.0332	0.1173	0.1011	0.0287	0.1252	0.0591	0.0199				
MZSH	0.0644	0.0964	0.0753	0.1557	0.0315	0.0762	0.0621	0.0310	0.0612	0.0327			
OAUR	0.3072	0.3518	0.3493	0.3676	0.2820	0.3066	0.2993	0.3034	0.3544	0.3324	0.3323		
OMOS	0.3029	0.3098	0.2757	0.3488	0.2707	0.2729	0.3305	0.2663	0.2893	0.3273	0.3156	0.4803	

OSHI, *O. shiranus*; OSQU, *O. squamipinnis*; OKAR, *O. karongae*; OLID, *O. lidole*; DMSH, *O. shiranus* pond Domasi; DMOK, *O. karongae* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storagepond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu; MZKB, *O. karongae* breeding pond Mzuzu; MZSH, *O. shiranus* pond Mzuzu; OAUR, *O. aureus*; OMOS, *O. mossambicus*.

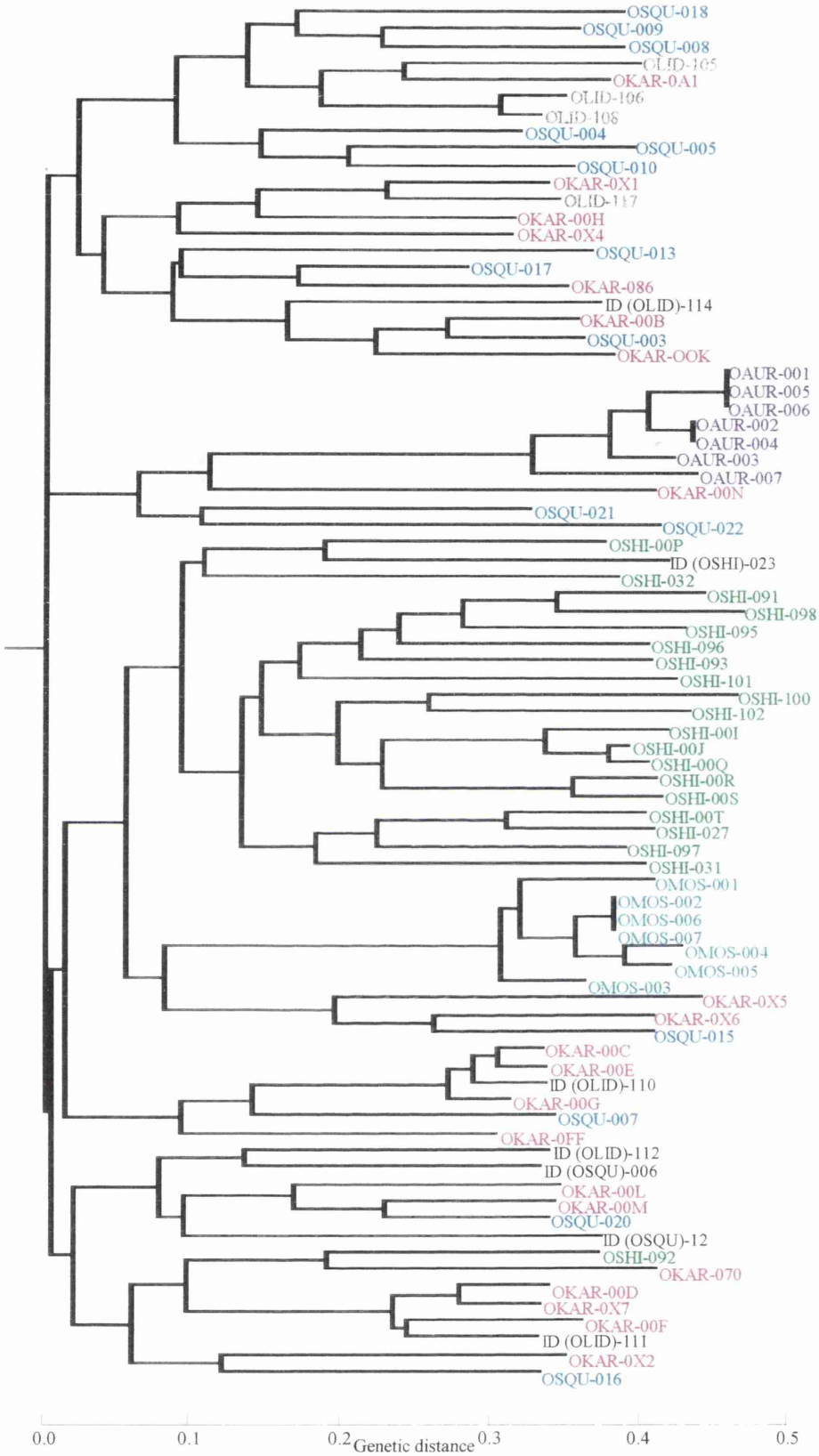

Figure III.4 RAPD-generated nearest neighbour-joining tree of wild fish from Malawi (OSHI, *Oreochromis shiranus;* OSQU, *O. squamipinnis;* OKAR, *O. karongae;* OLID, *O. lidole;* ID, Identification of fish unknown - suspected identity in parentheses) and two reference species (OAUR, *O. aureaus*; OMOS, *O. mossambicus*), based on the analyses of 43 RAPD fragments produced using the Primer OPA 08 only. All individuals labelled with identification number.

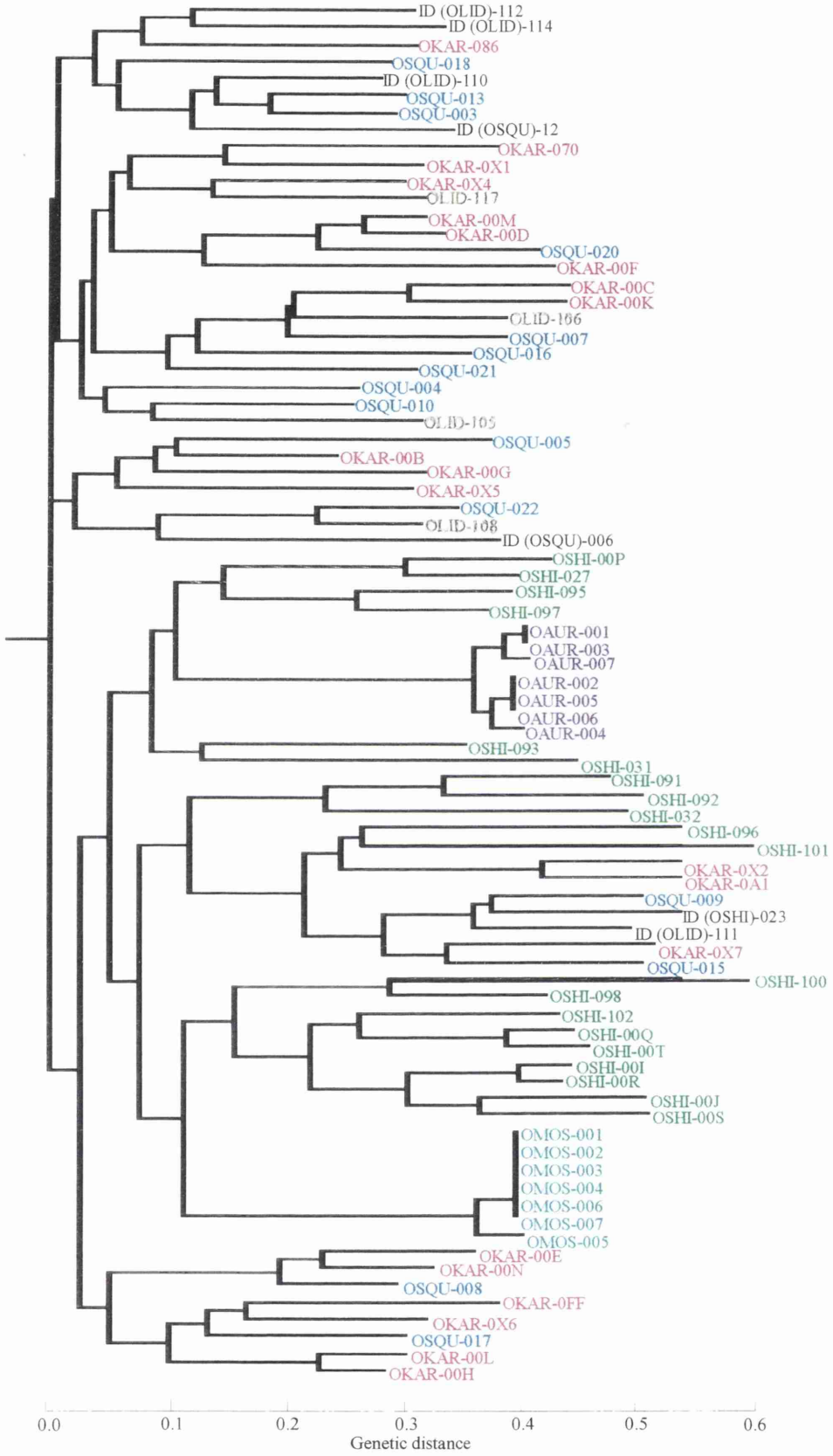

Figure III.5 RAPD-generated nearest neighbour-joining tree of wild fish from Malawi (OSHI, *Oreochromis shiranus;* OSQU, *O. squamipinnis;* OKAR, *O. karongae;* OLID, *O. lidole;* ID, Identification of fish unknown - suspected identity in parentheses) and two reference species (OAUR, *O. aureaus*; OMOS, *O. mossambicus*), based on the analyses of 57 RAPD fragments produced using the Primer OPA 10 only. All individuals labelled with identification number.

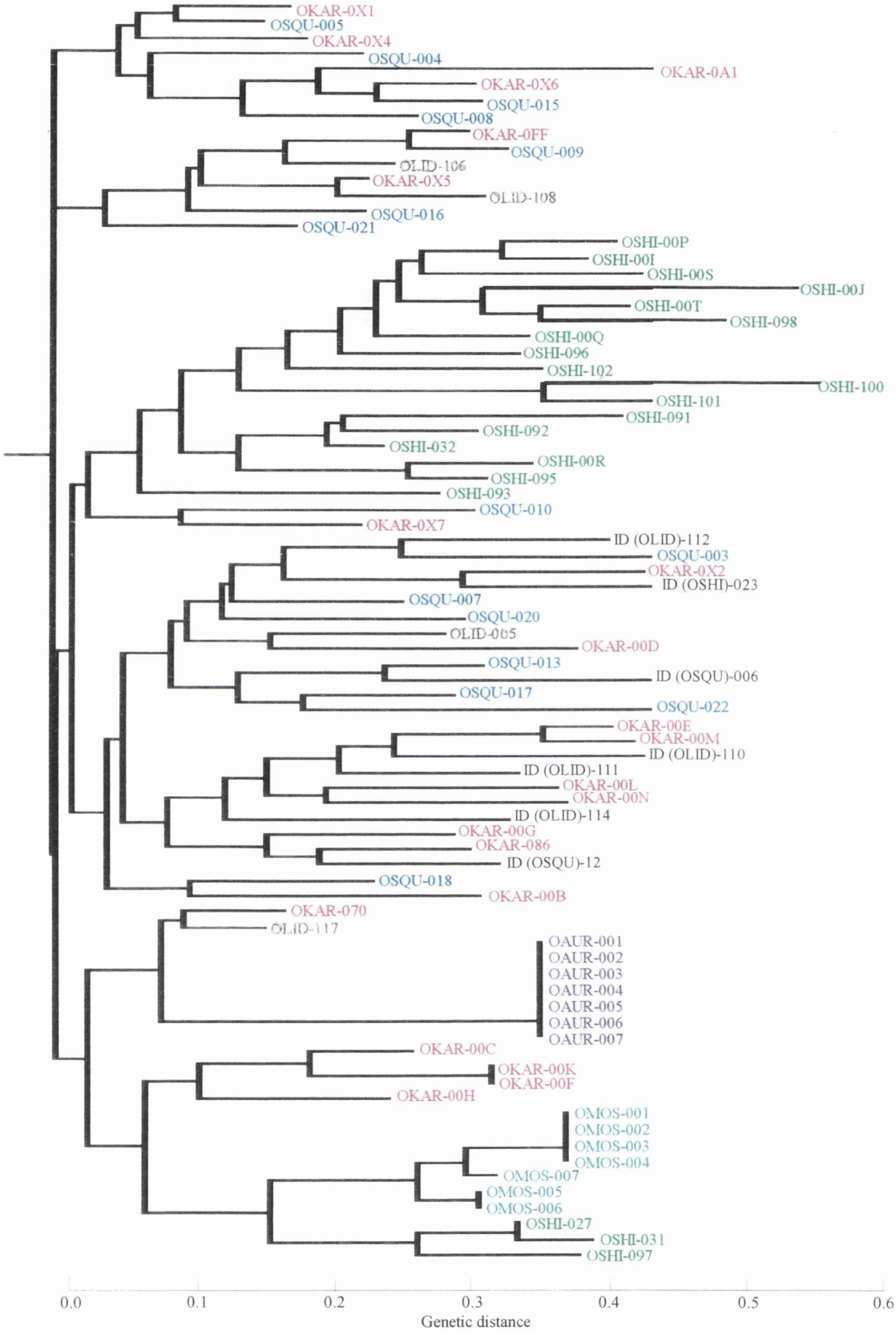

Figure III.6 RAPD-generated nearest neighbour-joining tree of wild fish from Malawi (OSHI, *Oreochromis shiranus;* OSQU, *O. squamipinnis;* OKAR, *O. karongae;* OLID, *O. lidole;* ID, Identification of fish unknown - suspected identity in parentheses) and two reference species (OAUR, *O. aureaus*; OMOS, *O. mossambicus*), based on the analyses of 21 RAPD fragments produced using the Primer OPE 15 only. All individuals labelled with identification number.

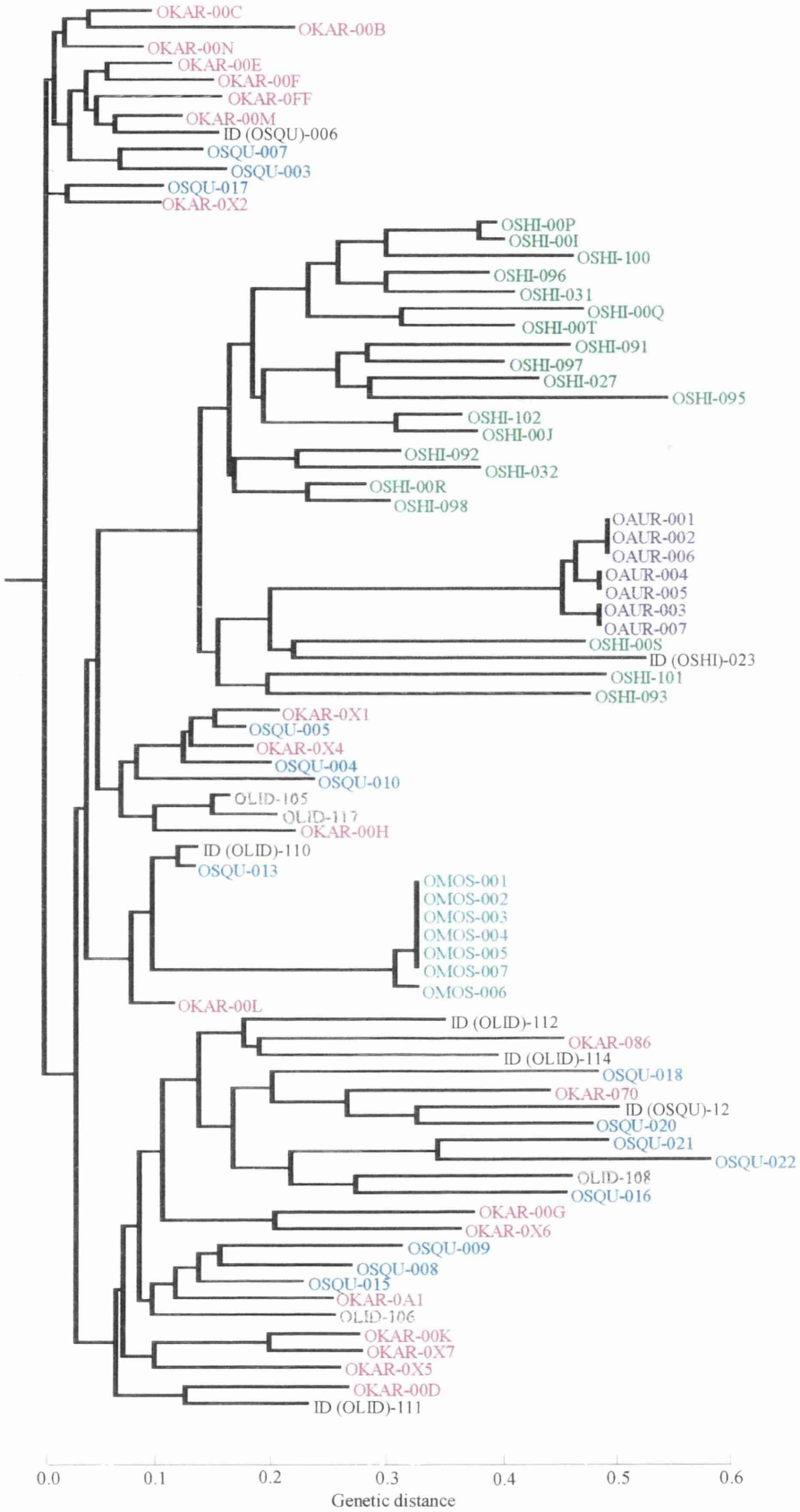

Figure III.7 RAPD-generated nearest neighbour-joining tree of wild fish from Malawi (OSHI, *Oreochromis shiranus;* OSQU, *O. squamipinnis;* OKAR, *O. karongae;* OLID, *O. lidole;* ID, Identification of fish unknown - suspected identity in parentheses) and two reference species (OAUR, *O. aureaus*; OMOS, *O. mossambicus*), based on the analyses of 42 RAPD fragments produced using the Primer OPG 17 only. All individuals labelled with identification number.

Table III.4 Measures of genetic divergence (Nei & Tajima 1981) between pairs of samples (100 individuals in total) used in RAPD analysis, but only those individuals used also in allozyme electrophoretic analysis. Values based on the analyses of only nine diagnostic RAPD fragments (above diagonal), and of all 163 RAPD fragments (below diagonal). Computed for comparisons with corresponding matrices obtained by allozyme analysis.

	OSHI n=12	OSQU n=15	OKAR n=2	OLID n=4	DMSH n=10	DMOK n =9	DWSE n=21	DWST n=17	MZKH n=10
OSHI		0.9291	0.9286	0.9421	0.0462	0.7826	0.0261	0.2513	0.8467
OSQU	0.1430		0.0742	-0.0049	0.6394	-0.0097	0.6797	0.3080	-0.0012
OKAR	0.1101	0.0394		0.0279	0.6397	0.0824	0.6736	0.2996	0.1211
OLID	0.1975	0.0188	0.0650		0.6576	0.0004	0.6916	0.3006	0.0253
DMSH	0.0018	0.1041	0.0762	0.1778		0.5150	-0.0018	0.0983	0.5743
DMOK	0.0985	0.0164	-0.0005	0.0613	0.0699		0.5465	0.2171	-0.0153
DWSE	0.0171	0.1162	0.1006	0.1788	0.0027	0.0942		0.1107	0.6120
DWST	0.0504	0.0447	0.0328	0.0999	0.0239	0.0357	0.0277		0.2944
MZKH	0.1283	0.0402	0.0420	0.0909	0.1051	0.0090	0.1318	0.0672	

OSHI, *O. shiranus*; OSQU, *O. squamipinnis*; OKAR, *O. karongae*; OLID, *O. lidole*; DMSH, *O. shiranus* pond Domasi; DMOK, *O. karongae* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storagepond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu; MZKB, *O. karongae* breeding pond Mzuzu; MZSH, *O. shiranus* pond Mzuzu.

Table III.5 Measures of genetic differentiation between pairs of samples (100 individuals in total) used in allozyme electrophoretic analysis (Nei's (1978) genetic distance),but only those used also in RAPD analysis. Computed for comparisons with corresponding matrices obtained by RAPD analysis.

	OSHI n=12	OSQU n=15	OKAR n=2	OLID n=4	DMSH n=10	DMOK n =9	DWSE n=21	DWST n=17	MZKH n=10
OSHI									
OSQU	0.3600								
OKAR	0.3980	0.0350							
OLID	0.3730	0.0060	0.0370						
DMSH	0.0310	0.3580	0.3760	0.3590					
DMOK	0.3270	0.0190	0.0560	0.0160	0.3050				
DWSE	0.0190	0.3380	0.3894	0.3610	0.0610	0.3150			
DWST	0.2770	0.2200	0.2400	0.2190	0.1830	0.2230	0.2720		
MZKH	0.3270	0.0210	0.0300	0.0210	0.3110	0.0150	0.3190	0.2290	

OSHI, *O. shiranus*; OSQU, *O. squamipinnis*; OKAR, *O. karongae*; OLID, *O. lidole*; DMSH, *O. shiranus* pond Domasi; DMOK, *O. karongae* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storagepond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu; MZKB, *O. karongae* breeding pond Mzuzu; MZSH, *O. shiranus* pond Mzuzu.

APPENDIX IV: MORPHOLOGY

Table IV.1 Means and standard deviations of measurements (original data) recorded for males only, and for males and females of the four pure species (from Lakes Malawi and Malombe), of all hybrid fish (from five farm ponds) and of hybrid fish from two of the farm ponds separately (DWSE and DWST). Maximum and minimum values in parenthesis.

		OSHI	OKAR	OLID	OSQU	HYBRID	DWSE	DWST
M*	♂	n=16	n=13	n=13	n=23	n=42	n=9	n=19
	♂ & ♀	n=21	n=24	n=18	n=23	n=51	n=13	n=23
S.L.	♂	190.6 ± 19.2 (136.0-218.0)	228.7 ± 39.6 (195.0-312.0)	266.1 ± 16.0 (231.0-291.0)	208.1 ± 17.4 (184.0-251.0)	127.9 ± 25.4 (78.0-184.0)	135.9 ± 13.3 (109.8-151.0)	131.8 ± 17.5 (95.0-160.0)
	♂ & ♀	192.0 ± 20.5 (136.0-229.0)	228.2 ± 32.8 (187.0-312.0)	274.9 ± 21.7 (231.0-318.0)		128.3 ± 23.9 (78.0-184.0)	134.9 ± 15.6 (109.8-166.0)	129.9 ± 17.0 (95.0-160.0)
B.D.	♂	77.1 ± 8.8 (52.6-94.6)	87.6 ± 14.3 (76.0-117.0)	100.2 ± 6.0 (89.6-110.0)	83.9 ± 7.3 (73.7-101.8)	48.8 ± 10.0 (27.3-66.5)	53.5 ± 6.3 (40.2-60.6)	51.3 ± 5.6 (39.6-59.0)
	♂ & ♀	76.8 ± 9.7 (21.9-94.6)	88.9 ± 12.9 (71.4-117.0)	104.1 ± 9.5 (89.6-125.2)		48.6 ± 9.2 (27.3-66.5)	52.4 ± 6.3 (40.2-60.6)	50.3 ± 5.8 (39.4-59.0)
C.D.	♂	26.7 ± 2.8 (19.1-31.9)	27 ± 4.0 (23.7-35.0)	31.2 ± 1.9 (26.5-34.6)	25.3 ± 2.1 (21.8-30.0)	16.8 ± 3.1 (9.7-21.1)	18.7 ± 2.1 (14.4-20.9)	17.4 ± 2.6 (10.8-20.5)
	♂ & ♀	26.7 ± 3.1 (19.2-31.9)	27.4 ± 3.5 (23.6-35.0)	32.1 ± 2.6 (26.5-38.8)		16.8 ± 2.9 (9.7-21.9)	18.5 ± 2.1 (14.4-21.9)	17.1 ± 2.5 (10.8-20.5)
C.L.	♂	24.7 ± 2.6 (18.2-27.6)	36.2 ± 5.9 (29.7-47.4)	43.8 ± 3.5 (37.2-49.9)	33.4 ± 3.1 (29.2-40.1)	17.0 ± 4.6 (9.5-28.6)	17.6 ± 1.3 (15.7-19.5)	16.6 ± 3.1 (9.5-22.5)
	♂ & ♀	24.8 ± 2.9 (18.0-28.1)	36.3 ± 5.0 (28.9-47.4)	44.4 ± 3.6 (37.2-49.9)		17.1 ± 4.4 (9.5-28.6)	17.7 ± 2.5 (15.7-24.7)	16.6 ± 3.1 (9.5-22.5)
H.L.	♂	60.9 ± 7.0 (40.9-72.1)	74.5 ± 12.8 (61.8-97.6)	93.9 ± 6.9 (80.3-101.9)	69.2 ± 6.3 (60.3-84.4)	42.5 ± 8.4 (27.0-58.5)	42.6 ± 4.4 (35.4-48.1)	45.2 ± 5.9 (31.6-53.5)
	♂ & ♀	61.7 ± 7.2 (40.9-73.5)	74.4 ± 11.1 (58.5-97.6)	95.8 ± 6.7 (80.3-103.7)		42.4 ± 7.8 (27.0-58.5)	42.3 ± 5.1 (35.4-52.4)	44.6 ± 5.8 (31.6-53.5)
I.W.	♂	24.7 ± 2.7 (16.7-29.4)	36.0 ± 7.3 (29.0-50.6)	46.2 ± 3.8 (39.4-51.9)	32.7 ± 3.5 (26.8-41.6)	15.4 ± 3.9 (8.2-24.1)	15.3 ± 1.7 (12.2-17.5)	16.7 ± 2.2 (12.6-20.3)
	♂ & ♀	25.0 ± 3.4 (16.7-32.3)	36.1 ± 6.1 (29.0-50.6)	47.2 ± 3.6 (39.4-51.9)		15.5 ± 3.7 (8.2 ± 24.1)	15.1 ± 2.0 (12.2-19.0)	16.4 ± 2.5 (11.1-20.3)
L.J.	♂	21.1 ± 3.2 (12.7-25.7)	23.0 ± 4.3 (18.8-31.0)	33.2 ± 3.4 (25.8-37.9)	21.4 ± 2.1 (17.8-26.2)	14.6 ± 3.2 (8.6-19.9)	15.2 ± 2.3 (11.7-18.3)	15.8 ± 3.2 (8.6-19.9)
	♂ & ♀	21.3 ± 3.2 (12.7-26.2)	22.9 ± 3.7 (17.4-31.0)	33.4 ± 3.0 (25.8-37.9)		14.4 ± 3.1 (8.6-19.9)	14.9 ± 2.6 (11.2-19.2)	15.4 ± 3.2 (8.6-19.9)
M.W.	♂	33.9 ± 3.4 (25.8-39.6)	41.0 ± 7.3 (32.3-57.9)	49.8 ± 4.4 (40.1-56.5)	37.9 ± 4.0 (30.2-46.4)	22.8 ± 4.5 (13.5-32.6)	25.1 ± 3.0 (18.9-29.1)	23.6 ± 2.6 (17.3-26.8)
	♂ & ♀	34.1 ± 3.9 (25.8-41.5)	40.9 ± 6.7 (31.9-57.9)	51.0 ± 4.4 (40.1-56.8)		22.9 ± 4.3 (13.5-32.6)	25.1 ± 3.5 (18.9-31.9)	23.3 ± 2.6 (17.3-26.8)
SN.L.	♂	22.9 ± 3.0 (14.7-28.2)	28.7 ± 6.5 (23.1-41.2)	37.8 ± 3.6 (30.6-42.9)	25.7 ± 2.7 (21.5-31.9)	14.3 ± 3.6 (8.3-20.8)	14.1 ± 2.1 (10.9-17.2)	15.2 ± 2.9 (9.4-19.7)
	♂ & ♀	22.9 ± 3.0 (14.7-28.2)	28.5 ± 5.4 (22.5-41.2)	38.7 ± 3.5 (30.6-43.0)		14.1 ± 3.4 (8.3-20.8)	13.8 ± 2.4 (10.9-17.9)	14.8 ± 2.9 (9.4-19.7)

* M, measurement: S.L., standard length; B.D., maximum body depth; C.D., caudal peduncle depth; C.L. caudal peduncle length; H.L., head length; I.W., interorbital width; L.J, lower jaw length; M.W., maximum body width; SN.L., snout length. ♂, males only; ♂ & ♀, males and females.

Table IV.2 Scores for each variable, and percentage of variance explained, for four of the five principal components (PC) obtained from a PCA on morphometric data (eight size-adjusted measurements), dentition and anal fin spine count of male only, and of male and female pure fish sampled from the wild and hybrid fish sampled from farm ponds in Malawi.

	Males and females (n=133)				Males only (n=105)			
Variable †	PC 1	PC 2	PC 3	PC 4	PC 1	PC 2	PC 3	PC 4
B.D.	-0.146	0.058	0.792	-0.294	-0.235	0.028	0.763	-0.312
C.D.	-0.551	0.446	0.493	-0.135	-0.574	0.469	0.474	-0.039
C.L.	0.435	-0.568	-0.061	0.079	0.370	-0.593	-0.154	0.027
H.L.	0.744	0.476	0.138	0.297	0.788	0.351	0.237	0.274
I.W.	0.796	-0.146	0.297	-0.062	0.769	-0.222	0.318	-0.176
L.J.	0.279	0.751	0.049	0.317	0.325	0.667	0.152	0.453
M.W.	0.301	0.452	0.440	-0.097	0.272	0.392	0.538	-0.224
SN.L.	0.550	0.467	0.036	0.435	0.571	0.390	0.055	0.469
3 fin spines	-0.717	0.489	-0.135	0.124	-0.680	0.542	-0.156	0.179
4 fin spines	0.717	-0.489	0.135	-0.124	0.680	-0.542	0.156	-0.179
3 teeth rows	-0.495	-0.547	0.357	0.208	-0.586	-0.554	0.263	0.232
4 teeth rows	-0.053	0.473	-0.358	-0.195	0.076	0.470	-0.277	-0.530
5 teeth rows	0.452	-0.023	0.068	0.141	0.424	-0.024	0.093	0.356
6 teeth rows	0.186	-0.006	0.015	-0.212	0.166	0.033	-0.041	0.108
Arrange 1	0.203	0.131	-0.728	-0.351	0.268	0.283	-0.715	-0.235
Arrange 2	-0.305	-0.265	0.261	0.785	-0.365	-0.378	0.234	0.532
Arrange 3	0.124	0.161	0.544	-0.522	0.100	0.098	0.586	-0.334
% var.	22.6	16.9	14.0	9.9	23.2	16.7	14.2	9.8

† Variables: B.D., maximum body depth; C.D., caudal peduncle depth; C.L. caudal peduncle length; H.L., head length; I.W., interorbital width; L.J., lower jaw length; M.W., maximum body width;SN.L., snout length; Teeth row arrangement: Arrange 1, Only the first row is distinct; Arrange 2, All rows distinct; Arrange 3, All rows indistinct and overlapping.
% var., Percentage of variance explained.

Table IV.3 Within-groups correlations for each measurement, percentage of variance explained and the significance of functions (Wilks' Lambda) for the four canonical discriminant functions (DF) obtained from a DFA on morphometric data (eight size-adjusted measurements), dentition and anal fin spine count of male only, and of male and female pure fish sampled from the wild and hybrid fish sampled from farm ponds in Malawi.

	Males and females (n=133)				Males only (n=105)			
Variable †	DF 1	DF 2	DF 3	DF 4	DF 1	DF 2	DF 3	DF 4
B.D.	-0.107	0.453	0.061	-0.329	-0.185	0.364	0.040	-0.404
C.D.	0.268	0.434	0.315	-0.378	0.144	0.453	0.319	-0.441
C.L.	-0.404	-0.089	-0.120	0.150	-0.339	-0.211	-0.059	0.077
H.L.	-0.065	-0.360	-0.010	-0.484	-0.002	-0.387	0.024	-0.446
I.W.	-0.452	-0.039	0.103	-0.174	-0.425	-0.236	0.124	-0.165
L.J.	0.166	-0.328	0.213	-0.658	0.211	-0.257	0.234	-0.578
M.W.	-0.051	-0.053	0.011	-0.260	-0.052	-0.055	0.057	-0.237
SN.L.	0.040	-0.278	-0.040	-0.307	0.125	-0.241	-0.009	-0.273
3 fin spines	0.635	0.228	-0.121	-0.144	0.485	0.380	-0.034	-0.275
4 fin spines	-0.635	-0.228	0.121	0.144	-0.485	-0.380	0.034	0.275
3 teeth rows	-0.002	0.414	-0.282	0.460	-0.080	0.361	-0.312	0.364
4 teeth rows	0.109	-0.016	0.297	-0.264	0.061	0.031	0.327	0.010
5 teeth rows	-0.114	-0.324	-0.074	0.099	0.004	-0.322	-0.052	-0.063
6 teeth rows	-0.036	-0.062	-0.072	-0.322	-0.013	-0.106	-0.132	-0.481
Arrange 1	0.100	-0.323	0.550	0.475	0.205	-0.257	0.456	0.379
Arrange 2	-0.005	0.162	-0.509	-0.055	-0.089	0.173	-0.382	0.077
Arrange 3	-0.093	0.140	0.020	-0.410	-0.107	0.068	-0.040	-0.458
% var.	52.29	30.18	13.11	4.13	47.55	33.68	14.97	3.81
Wilks'	0.032 ***	0.143 ***	0.425 ***	0.788 **	0.019 ***	0.092 ***	0.344 ***	0.764 *

† Variables: B.D., maximum body depth; C.D., caudal peduncle depth; C.L. caudal peduncle length; H.L., head length; I.W., interorbital width; L.J., lower jaw length; M.W., maximum body width; SN.L., snout length; Teeth row arrangement: Arrange 1, Only the first row is distinct; Arrange 2, All rows distinct; Arrange 3, All rows indistinct and overlapping.

3 variables failed the tolerance test for DFA : 4 fin rays, 6 teeth rows and Arrange 3.

% var., Percentage of variance explained.

Wilks', Wilks' Lambda and its' significance for current and remaining functions, where *** $P<0.001$; ** $P<0.005$ and * $P<0.05$.

Table IV.4 Within-groups correlations for each variable, percentage of variance explained and the significance of functions (Wilks' Lambda) for the three canonical discriminant functions (DF) obtained from a DFA on morphometric data (eight size-adjusted measurements) and dentition data (teeth row count and arrangement) of male only, and of male and female pure fish sampled from the wild. This DFA did not include hybrid fish, which were treated as "ungrouped" cases.

	Male and females (n=86)			Males only (n=65)		
Variable †	DF 1	DF 2	DF 3	DF 1	DF 2	DF 3
B.D.	0.044	-0.217	-0.191	0.023	-0.263	-0.277
C.D.	-0.407	-0.348	-0.060	-0.481	-0.327	-0.194
C.L.	0.435	0.257	0.017	0.403	0.244	0.007
H.L.	0.040	0.434	-0.371	0.090	0.441	-0.411
I.W.	0.399	0.416	-0.069	0.400	0.384	-0.104
L.J.	-0.293	0.458	-0.381	-0.255	0.410	-0.427
M.W.	0.024	0.108	-0.158	0.026	0.092	-0.145
SN.L.	-0.049	0.270	-0.280	-0.061	0.249	-0.280
3 teeth rows	0.111	-0.691	0.163	0.109	-0.634	0.220
4 teeth rows	-0.171	0.123	0.014	-0.145	0.107	0.140
5 teeth rows	0.106	0.219	0.016	0.073	0.196	-0.073
6 teeth rows	0.024	0.073	-0.231	0.043	0.047	-0.328
Arrange 1	-0.167	0.390	0.668	-0.224	0.390	0.471
Arrange 2	0.109	-0.360	-0.357	0.146	-0.319	-0.095
Arrange 3	0.048	0.016	-0.287	0.061	-0.032	-0.366
% var.	65.41	25.40	9.19	57.43	33.95	8.62
Wilks'	0.023 ***	0.160 ***	0.539 ***	0.011 ***	0.093 ***	0.484 ***

† Variables: B.D., maximum body depth; C.D., caudal peduncle depth; C.L. caudal peduncle length; H.L., head length I.W., interorbital width; L.J., lower jaw length; M.W., maximum body width; SN.L., snout length; Teeth row arrangement: Arrange 1, Only the first row is distinct; Arrange 2, All rows distinct; Arrange 3, All rows indistinct and overlapping.

2 variables failed the tolerance test for DFA: 6 teeth rows and Arrange 3.

% var., Percentage of variance explained.

Wilks', Wilks' Lambda and its' significance for current and remaining DFs, where *** $P<0.001$.

Table IV.5 Within-groups correlations for each variable, percentage of variance explained and the significance of functions (Wilks' Lambda) for four of the five canonical discriminant functions (DF) obtained from DFA on morphometric data (eight size-adjusted measurements), dentition and anal fin spine count, of male pure fish sampled from the wild (four species) and fish sampled from two farm ponds at Domasi (DMOK and DMSH) in Malawi.

Variable †	DF 1	DF 2	DF 3	DF 4
B.D.	-0.032	0.595	0.260	-0.432
C.D.	0.255	0.523	0.375	-0.205
C.L.	-0.285	-0.182	0.072	-0.110
H.L.	-0.068	-0.305	0.372	-0.574
I.W.	-0.420	0.004	0.632	-0.297
L.J.	0.132	-0.404	0.349	-0.406
M.W.	-0.042	0.047	0.240	-0.227
SN.L.	-0.025	-0.133	0.579	-0.440
3 fin spines	0.480	0.391	0.213	0.118
4 fin spines	-0.480	-0.391	-0.213	-0.118
3 teeth rows	-0.023	0.414	-0.318	0.217
4 teeth rows	0.038	-0.051	0.237	0.127
5 teeth rows	-0.026	-0.203	-0.001	-0.123
6 teeth rows	-0.002	-0.083	-0.066	-0.332
Arrange 1	0.043	-0.346	0.317	0.482
Arrange 2	-0.014	0.213	-0.281	-0.128
Arrange 3	-0.025	0.091	0.011	-0.311
% var.	82.94	9.35	4.72	1.97
Wilks'	0.001 ***	0.020 ***	0.110 ***	0.353 ***

† Variables: B.D., maximum body depth; C.D., caudal peduncle depth; C.L. caudal peduncle length; H.L., head length; I.W., interorbital width; L.J., lower jaw length; M.W., maximum body width; SN.L., snout length; Teeth row arrangement: Arrange 1, Only the first row is distinct; Arrange 2, All rows distinct; Arrange 3, All rows indistinct and overlapping.

3 variables failed the tolerance test for DFA: 4 anal fin spines, 6 teeth rows and Arrange 2.

% var., Percentage of variance explained.

Wilks', Wilks' Lambda and its' significance for current and remaining DFs, where *** $P<0.001$; ** $P<0.005$

Table IV.6 Colouration recorded for the body (excluding head and fins), caudal fin margin, dorsal fin tips and dorsal fin lappets - expressed as the percentage of cases with a particular colour, in each group of pure species . The percentage of cases with a 'face mask' are also included.

	OSHI		OKAR		OLID		OSQU
	♂	♀	♂	♀	♂	♀	♂
	n=16	n=5	n=13	n=11	n=13	n=5	n=23
Body colour							
black (♂)	37.5	0.0	7.7	0.0	76.9	0.0	0.0
copper	0.0	0.0	92.3 *	100.0 †	0.0	0.0	0.0
dark grey	0.0	0.0	0.0	0.0	15.4	0.0	13.0
grey	18.7	20.0	38.5	0.0	37.7	40.0	13.0
olive	87.5	80.0	15.4	9.1	0.0	0.0	0.0
silver	0.0	0.0	46.1	0.0	0.0	100.0	100.0
golden yellow	43.7 *	100.0 †	0.0	0.0	0.0	0.0	0.0
Caudal margin							
no colour ♯	18.7	60.0	76.9	81.8	0.0	100.0	87.0
orange/red	81.3 *	40.0 †	0.0	0.0	0.0	0.0	0.0
white (♂)	0.0	0.0	23.1	0.0	100.0	0.0	13.0
yellow (♀)	0.0	0.0	0.0	18.2 †	0.0	0.0	0.0
Dorsal fin tips							
no colour ♯ (♀)	0.0	0.0	0.0	9.1	0.0	100.0	0.0
orange/red	100.0 *	100.0 †	0.0	0.0	0.0	0.0	0.0
white (♂)	0.0	0.0	15.4	0.0	100.0	0.0	100.0
yellow	0.0	0.0	84.6 *	90.9 †	0.0	0.0	0.0
Dorsal lappet							
translucent	31.2	20.0	7.7	0.0	0.0	20.0	0.0
red (♂)	56.3 *	0.0	0.0	0.0	0.0	0.0	0.0
no colour ♯	12.5	80.0	53.8	100.0	0.0	80.0	0.0
white (♂)	0.0	0.0	38.5	0.0	100.0	0.0	100.0
Face mask (♂)	0.0	0.0	0.0	0.0	0.0	0.0	100.0 *

OSHI, *O. shiranus*; OKAR, *O. karongae*; OLID, *O. lidole*; OSQU, *O. squamipinnis*.
♯ no colour, colour of caudal fin margin and dorsal fin tip and lappet indistinct from the rest of the fin colour;
(♂), Character found in males only; * Character unique to particular species for males of the pure species;
(♀), Character found in females only; † Character unique to particular species for females of the pure species.

Table IV.7 Within-groups correlations for each variable, percentage of variance explained and the significance of functions (Wilks' Lambda) for the three canonical discriminant functions (DF) obtained from a DFA on all morphological variables available, of male and female pure fish sampled from the wild. Used to classify eleven 'unknown' wild fish.

Variable †	DF 1	DF 2	DF 3
B.D.	-0.001	0.047	-0.120
C.D.	-0.152	-0.010	-0.102
C.L.	0.157	0.023	0.057
H.L.	0.028	0.038	0.267
I.W.	0.152	0.025	0.159
L.J.	-0.078	0.005	0.353
M.W.	0.019	0.011	0.130
SN.L.	-0.005	0.022	0.205
3 teeth rows	-0.007	-0.020	-0.404
4 teeth rows	-0.051	-0.024	0.094
5 teeth rows	0.047	0.005	0.099
6 teeth rows	-0.007	0.035	0.053
Arrange 1	-0.023	-0.142	0.187
Arrange 2	-0.012	0.085	-0.185
Arrange 3	-0.007	0.059	0.006
black b.c.	0.052	0.011	-0.191
copper b.c.	-0.276	0.597	0.479
dark grey b.c.	-0.015	-0.037	-0.051
grey b.c.	0.003	0.013	0.006
olive b.c.	0.182	0.026	0.227
silver b.c.	-0.120	-0.161	0.034
g. yellow b.c.	0.132	-0.005	0.134
no c.f.m.	-0.083	-0.015	0.146
red c.f.m.	0.181	-0.007	0.184
white c.f.m.	-0.014	0.006	-0.335
yellow c.f.m.	-0.017	0.037	0.030
no d.f.t.	-0.001	0.016	-0.142
white d.f.t.	-0.108	-0.213	-0.284
yellow d.f.t.	0.140	0.257	0.516
clear d.f.l.	0.056	0.008	0.039
no d.f.l.	-0.018	0.120	0.064
red d.f.l.	0.009	-0.004	0.101
white d.f.l.	-0.103	-0.152	-0.189
% var.	56.51	35.13	8.37
Wilks'	0.000 ***	0.000 ***	0.000 ***

† Variables: B.D., maximum body depth; C.D., caudal peduncle depth; C.L. caudal peduncle length; H.L., head length; I.W., interorbital width; L.J., lower jaw length; M.W., maximum body width; SN.L., snout length; Teeth row arrangement: Arrange 1, Only the first row is distinct; Arrange 2, All rows distinct; Arrange 3, All rows indistinct and overlapping; b.c., body colour; c.f.m., caudal fin margin; d.f.t., dorsal fin tips; d.f.l., dorsal fin lappet.

The variables of fin spine counts, face mask presence or absence and red dorsal fin tips could not be used in this DFA because they were fixed in each species. Four variables failed the tolerance test for DFA: 6 teeth rows, white dorsal lappets, yellow caudal fin margin and yellow dorsal fin tips.

% var., Percentage of variance explained. Wilks', Wilks' Lambda and significance for current and remaining DFs, where *** $P<0.001$.

Table IV.8 Colouration recorded for body (excluding fins and head), caudal fin margin, dorsal fin tips and dorsal fin lappets - expressed as the percentage of cases with a particular colour, in each farm pond sampled (for all fish; hybrids and 'pure' fish). The percentage of cases with a 'face mask' are also included.

	DMOK	DMSH	DWSE		DWST		MZKH	
	♂	♂	♂	♀	♂	♀	♂	♀
	n=10	n=10	n=11	n=4	n=21	n=4	n=7	n=3
Body colour								
black (♂)	60.0	10.0 *	36.4	0.0	47.6	0.0	0.0	0.0
copper	0.0	0.0	0.0	0.0	33.3	100.0	100.0	100.0
dark grey	30.0	70.0	27.3	0.0	36.1	0.0	28.6	0.0
grey	0.0	30.0	72.7	100.0	47.6	50.0	28.6 *	0.0
olive	0.0	30.0	28.2	25.0	47.6	50.0	85.7	100.0
silver	10.0 *	0.0	45.4	75.0	0.0	0.0	14.3 *	33.3 *
golden yellow	0.0	0.0	0.0	25.0	0.0	0.0	0.0	0.0
Caudal margin								
no colour ♯	90.0	0.0	36.4	100.0	14.3	25.0	42.9	100.0
orange/red	10.0	100.0	63.5	0.0	80.8	75.0	0.0	0.0
white (♂)	0.0	0.0	0.0	0.0	4.8 *	0.0	57.1	0.0
yellow (♀)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dorsal fin tips								
no colour ♯ (♀)	0.0	0.0	0.0	25.0	4.8	25.0	0.0	33.3 *
orange/red	0.0	100.0	100.0	75.0	80.9	75.0	71.4	0.0
white (♂)	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
yellow	0.0	0.0	0.0	0.0	14.3	0.0	28.6	66.7
Dorsal lappet								
translucent	10.0 *	0.0	81.8	25.0	52.4	0.0	42.8 *	0.0
red (♂)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
no colour ♯	0.0	0.0	18.2	75.0	42.8	100.0	28.6	100.0
white (♂)	90.0	100.0	0.0	0.0	4.8	0.0	28.6	0.0
Face mask (♂)	30.0 *	0.0	0.0	0.0	0.0	0.0	0.0	0.0

DMOK, *O. karongae* pond Domasi; DMSH, *O. shiranus* pond Domasi; DWSE, Sewage pond Dwangwa; DWST, Storage pond Dwangwa; MZKH, *O. karongae* holding pond Mzuzu.
♯ no colour, colour of caudal fin margin and dorsal fin tip and lappet indistinct from the rest of the fin colour;
(♂), Character found in males only; (♀), Character found in females only.
*, Character not found in individuals identified as hybrids with genetic analyses.

Table VI.9a Identification of individual farm fish from DMOK, DMSH and DWSE (hybrids and 'pure' fish based on genetic analyses) based on species-specific colouration only (I.D. 1) (See Table IV.8, and text of Chapter 5) and on colouration combined with anal fin spine count (I.D. 2). Species, or groups of species, associated with each characteristic are indicated in parenthesis below each character.

		Species-specific colouration characteristics **											
		Body colour		Caudal fin		Dorsal fin tip colour			Lappet		No. anal fin spines		
Farm pond	Fish no.	copper (OKAR)	yellow (OSHI)	red (OSHI)	white (CH)	red (OSHI)	white (CH)	yellow (OKAR)	white (CH)	I.D. 1 *	3 (CH)	4 (SH)	I.D. 2 †
DMOK	33 ×						✓		✓	CH	✓		
	34 ×						✓		✓	CH	✓		
	35 ×						✓		✓	CH+	✓		
	36						✓		✓	CH	✓		
	37 ×						✓		✓	CH	✓		
	38 ×						✓		✓	CH	✓		
	39 ×						✓			CH	✓		
	40 ×						✓		✓	CH	✓		
	41 ×						✓		✓	CH+	✓		
	42			✓			✓		✓	HY+	✓		
DMSH	43			✓		✓			✓	HY		✓	
	44			✓		✓			✓	HY		✓	
	45 ×			✓		✓			✓	HY		✓	
	46			✓		✓			✓	HY	✓		
	47			✓		✓			✓	HY		✓	
	48			✓		✓			✓	HY		✓	
	49			✓		✓			✓	HY		✓	
	50			✓		✓			✓	HY	✓		
	51			✓		✓			✓	HY		✓	
	52			✓		✓			✓	HY		✓	
DWSE	118		✓			✓				OSHI		✓	
	119 ×			✓		✓				OSHI		✓	
	120			✓		✓				OSHI		✓	
	121			✓		✓				OSHI		✓	
	122			✓		✓				OSHI		✓	
	123					✓				OSHI		✓	
	124			✓		✓				OSHI	✓		HY
	125					✓				OSHI		✓	
	126 ×					✓				OSHI		✓	
	127					✓				OSHI	✓		HY
	128			✓		✓				OSHI		✓	
	129					✓				OSHI		✓	
	130									?		✓	OSHI
	131					✓				OSHI	✓		HY
	132			✓		✓				OSHI		✓	

Fish no., identification number of fish; ×, those fish not identified as hybrids with genetic analysis.

** Coding for 'species' with particular characteristic: OKAR, *O. karongae*; OSHI, *O. shiranus*; CH, all chambo species; OLID, *O. lidole*; OSQU, *O. squamipinnis*.

* I.D. 1 - Identification 1 based on species-specific colouration only: CH, chambo species; HY, hybrid; OSHI; *O. shiranus;* +, individuals with a face mask; ?, Identity cannot be determined by characters used.

† I.D. 2 - Identification 2 based on colouration and anal fin spine count. Only those individuals where their identity is different from I.D. 1 are indicated (coding as for I.D. 1).

Table VI.9b Identification of individual farm fish from DWST, and MZKH (hybrids and 'pure' fish based on genetic analyses) based on species-specific colouration only (I.D. 1) (See Table IV.6) and on colouration combined with anal fin spine count (I.D. 2). Species, or groups of species, associated with each characteristic are indicated in parenthesis below each character.

		Species-specific colouration characteristics **									No. anal fin spines		
		Body colour		Caudal fin		Dorsal fin tip colour			Lappet				
Farm pond	Fish no.	copper (OKAR)	yellow (OSHI)	red (OSHI)	white (CH)	red (OSHI)	white (CH)	yellow (OKAR)	white (CH)	I.D. 1 *	3 (CH)	4 (SH)	I.D. 2 †
DWST	148			✓		✓				OSHI	✓		HY
	149	✓		✓		✓				HY	✓		
	150	✓		✓		✓				HY	✓		
	151			✓		✓				OSHI	✓		HY
	152			✓		✓				OSHI	✓		HY
	153			✓		✓				OSHI	✓		HY
	154	✓		✓		✓				HY		✓	
	155							✓		CH	✓		
	156	✓								CH	✓		
	157			✓		✓				OSHI	✓		HY
	158 ✕				✓			✓		CH	✓		
	159 ✕	✓						✓		CH	✓		
	160			✓		✓				OSHI		✓	
	161			✓		✓			✓	HY		✓	
	162			✓		✓				OSHI	✓		HY
	163			✓		✓				OSHI	✓		HY
	164	✓		✓		✓				HY	✓		
	165	✓		✓		✓				HY	✓		
	166			✓		✓				OSHI	✓		HY
	167	✓								CH	✓		
	168			✓		✓				OSHI	✓		HY
	169	✓		✓		✓				HY	✓		
	170	✓		✓		✓				HY	✓		
	171			✓		✓				OSHI	✓		HY
	172	✓		✓		✓				HY	✓		
MZKH	232 ✕	✓			✓	✓				HY	✓		
	233	✓				✓				HY	✓		
	234	✓				✓				HY	✓		
	235	✓			✓	✓				HY	✓		
	236 ✕	✓						✓		CH	✓		
	237 ✕	✓			✓			✓	✓	CH	✓		
	238	✓				✓				HY	✓		
	239 ✕	✓						✓	✓	CH	✓		
	240 ✕	✓			✓			✓	✓	CH	✓		
	241 ✕	✓								CH	✓		

Fish no., identification number of fish; ✕, those fish not identified as hybrids with genetic analysis.

** Coding for 'species' with particular characteristic: OKAR, *O. karongae*; OSHI, *O. shiranus*; CH, all chambo species; OLID, *O. lidole*; OSQU, *O. squamipinnis*.

* I.D. 1 - Identification 1 based on species-specific colouration only: CH, chambo species; HY, hybrid; OSHI; *O. shiranus*.

† I.D. 2 - Identification 2 based on colouration and anal fin spine count. Only those individuals where their identity is different from I.D. 1 are indicated (coding as for I.D. 1).